Third Edition

Animal Models in Toxicology

Edited by

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Preface

Biomedical sciences use animals as models to understand and predict responses in humans; in toxicology and pharmacology, in particular, this remains both the major tool for biomedical advances and the source of significant controversy. On the one hand, animal models have provided the essential components for research and serve as the source that has permitted the explosive growth of understanding in these fields, with the multitude of benefits to both humans and other animal species. New technologies make both the scope and quality of potential information from these experiments an ever-expanding set of dimensions. At the same time, the benefits of such use, balanced against costs in terms of animal lives, potential suffering, and discomfort, must remain an essential part of the consideration in their use. These themes and factors in its resolution run through this third edition of *Animal Models in Toxicology*.

The cost–benefit discussion inherent in animal use has provided a continuous stimulus for significant and continuous advances in the technology employed in the humane use of animals and understanding of the relevance of findings to what may happen in similarly exposed people. These advances are reflected in this new edition. Every section has been updated, and more guidance has been directed to specific uses of animals in toxicology units.

Scientists have used animal models for so long that there is truth in the belief that many researchers employ animals primarily out of habit, with little or no thought as to what the best tools are and the optimum way of using them. At the same time, while there are elements of poor practice that are real, by and large, animals have worked exceptionally well as predictive models for humans—when properly used (Gad, 1990 and Chapters 13 and 14).

Regulations governing the purchase, husbandry, and use of animals in research have continued to change over the course of the twenty-first century. Indeed, in some countries (and even cities in the United States), such use has been banned for some purposes (cosmetics) and some sources of animals made unavailable.

The real and most apparent problems underlying the failures of animal models to accurately predict what will happen in humans arise primarily from selecting the wrong model, in not using an animal model correctly, or in poorly extrapolating results to humans. Increasingly, graduate degree programs in the biomedical sciences do not currently address these issues well (if at all) in their curricula. Indeed, broad training in animal model selection and use, and the techniques involved in such research, currently has limited availability and where/when available is not well utilized. This text originally was developed to address these needs. Indeed, it is essential to the performance of good science that the correct species be used as a model and that data be analyzed appropriately.

Chapter 1 presents a historical review of the use of animal models and an overview of broad considerations of metabolism and relevance to use in toxicology. The core of the book, however, is in Chapters 2 through 10. Each of these chapters represents the joint efforts of experts in toxicology (addressing techniques for animal use and husbandry and peculiarities of the species as a toxicological model), toxicological pathology, and species-specific metabolism. For an investigator who is not well versed in the use of a particular species, each of these chapters provides an excellent introductory "course," along with guidance to the literature for more detailed understanding. All the major species used (and strains or breeds within these species) are addressed in these core chapters, and all of the chapters have been thoroughly updated since the second edition.

Chapter 11 presents the case for a range of alternative species (fish, earthworms, etc.) not commonly used for safety assessment studies but which may provide useful alternative models for some specific endpoints.

In Chapter 12, Niraj K. Tripathi and Robert Hall discusses the special considerations regarding the evaluation and interpretation of clinical pathology of the major model species.

Chapter 16 addresses, in detail, the general case of how to select a model species and how to extrapolate the results to humans. Chapter 17 details the pitfalls in the process—the situations that cause either the human or the model to be significantly more sensitive than the other or totally irrelevant to each other in specific cases.

Chapter 18 presents an overview of the regulations that govern how laboratory animals are obtained, maintained, and utilized. Such laws have become increasingly complex, and an understanding of what can (and cannot) be done is essential for the modern researcher.

New to this edition are five additional chapters. The first of these (Chapter 19) provides specific and detailed guidance on the humane, efficient, and effective performance of necropsies for laboratory animals. The last three add detailed presentations of available new technologies for laboratory animal research—imaging technologies (Chapter 14), use of telemetry in animal studies (Chapter 13), transgenic animal models (Chapter 15), and immunology endpoint for assessment (Chapter 20).

The Appendix provides a quick guide to the major commercial sources of laboratory animals, whether common (rats and mice) or harder to come by (Chinese hamsters and primates). Information on the selection and use of common anesthetics, drugs, and pharmacological agents for use in laboratory animals is available in Borchard's *Drug Dosage In Laboratory Animals: A Handbook* (Telford Press, Telford, PA).

Our aim in this book is to provide a single-source reference for the use of animal models in toxicology.

REFERENCE

Gad, S. C. (1990) *Animal Models in Toxicology*. Marcel Dekker, New York.

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Shayne Cox Gad, PhD, DABT, has been the principal of Gad Consulting Services in Cary, North Carolina, since 1993. He has more than 37 years of broad-based experience in toxicology, drug and device development, document preparation, statistics, and risk assessment, having previously been the director of toxicology and pharmacology for Synergen (Boulder, Colorado), director of medical affairs technical support system services for Becton Dickinson (Research Triangle Park, North Carolina), and senior director of product safety and pharmacokinetics for G.D. Searle (Skokie, Illinois). He is past president of the American College of Toxicology, and a board-certified toxicologist (DABT). He is also a member of the Society of Toxicology, the Teratology Society, the Society of Toxicological Pathology, the Biometrics Society, and the American Statistical Association.

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Introduction

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BACKGROUND

The use of animals in experimental medicine, pharmacology, pharmaceutical and medical device development, safety assessment, and toxicological evaluation is a well-established, regulatorily required, and essential practice. Whether serving as a source of isolated organelles, cells, or tissues, a disease model, or a predictive model for drug or other xenobiotic action or transformation in man, animal models have provided the necessary building blocks that have permitted the explosive growth of medical and biological knowledge in the later half of the twentieth century and into the

twenty-first century (Meier and Stocker, 1989; Nevalainen et al., 1996). Animal experiments also have served rather successfully as identifiers of potential hazards, benefits, and toxicity in humans for synthetic chemicals and biologicals with many intended uses.

Animals have been used as models for centuries to predict what chemicals and environmental factors would do to humans. The earliest uses of experimental animals are lost in prehistory, though much of what is recorded in early history about toxicology testing indicates that humans were the model of choice. The earliest clear description of the use of animals in the scientific study of the effects of environmental agents appears to be by Priestley (1792) in his study of gases. The first systematic use of animals for the screening of a wide variety of agents was published by Orfila (1814) and was described by Dubois and Geiling (1959) in their historical review. This work consisted of dosing test animals with known quantities of agents (poisons, drugs, or biologic agents) and included the careful recording of the resulting clinical signs and gross necropsy observations. The use of animals as predictors of both potential ill and beneficial effects has grown since that time.

CURRENT ANIMAL STUDIES

The current regulatorily required use of animal models in acute testing began by using them as a form of instrument to detect undesired contaminants when electronic chemical or mechanical detectors were unavailable. For example, miners used canaries to detect the presence of carbon monoxide, a case in which an animal model is more sensitive than humans (Burrell, 1912). In 1907, the U.S. Food and Drug Administration started to protect the public by the use of a voluntary testing program for new coal tar colors in foods. This was replaced by a mandatory program of testing in 1938, and such regulatorily required animal testing programs have continued to expand though for the last two decades there has been more thoughtful consideration of such uses.

The knowledge gained by experimentation on animals has undoubtedly increased both the length and quality of our lives, an observation that most reasonable people would find difficult to dispute, but it (as reviewed by Ewald and Gregg, 1983) has also benefited animals. As is the case with many tools, animals have sometimes been used inappropriately. These unfortunate instances have helped fuel the actions of a vituperative animal "rights" movement. This movement has encouraged a measure of critical self-appraisal on the part of scientists concerning the issues of the care and usage of animals. The Society of Toxicology and the American College of Toxicology both established Animals in Research Committees in the 1980s, and these have published guidelines for the use of animals in research and testing. In general, the purpose of these committees is to foster thinking on the four Rs of animal-based research: reduction, refinement, (research into) replacements, and responsible use. This third edition is, in part, a response to these continued concerns.

The media frequently carry reports that state that most (if not at all) animal testing and research is not predictive of what will happen in people, and therefore, such testing is unwarranted. Many of the animal rights groups also present this argument at every opportunity and reinforce it with examples that entail seemingly great suffering in animals but that add nothing to the health, safety, and welfare of society (i.e., Fano, 1998). This is held to be especially the case for safety testing and research in toxicology. Animal rights activists try to "prove" this point by presenting reported and real examples of failure (such as thalidomide). In light of the essential nature of animal research and testing in toxicology, this is equivalent to seeking to functionally disarm us as scientists and as a society. Our primary responsibility (the fourth R) is to provide the information to protect people and the environment, and without animal models we cannot discharge this responsibility.

When confronted with this argument, all too many toxicologists cannot respond with examples to the contrary. Indeed, many might not even fully understand the argument at all. Very few are familiar enough with some of the history of toxicity testing to be able to counter with examples where it has not only accurately predicted a potential hazard to humans, but where research has directly

benefited both people and animals. There are, however, many such examples. Demonstrating the actual benefit of toxicology testing and research with examples that directly relate to the everyday lives of most people and not esoteric, basic research findings (which are the most exciting and interesting products to most scientists) are not an easy task. Examples that can be seen to affect neighbors, relatives, and selves on a daily basis would be the most effective. The problem is that toxicology is, in a sense, a negative science. The things we find and discover are usually adverse. If the applied end of our science works correctly, then the results are things that do not happen, and therefore are not seen.

If we correctly identify toxic agents (using animals and other predictive model systems) in advance of a product or agent being introduced into the marketplace or environment, generally it will not be introduced (or it will be removed), and society will not see death, rashes, cardiovascular, renal and hepatic diseases, cancer, or birth defects, for example. As these things already occur at some level in the population, it would seem that seeing less of them would be hard to firmly tie to the results of toxicity testing that rely on animals. In addition, the fact that animals are predictive models for man remains, as all scientific hypothecs, constantly open to revision (and refinement).

ORIGINS OF PREDICTIVE ANIMAL TESTING

The actual record of evidence for the predictive value of animal studies and how they have benefited man and domestic animals will be reviewed in the following two sections. However, the negative image needs to be rebutted. First, it must be remembered that predictive animal testing in toxicology, as we now know it, arose largely out of three historical events.

"Lash Lure" Case

Early in the 1930s, an untested eyelash dye containing p-phenylenediamine (Lash Lure) was brought onto the market in the United States. This product (as well as a number of similar products) rapidly demonstrated that it could sensitize the external ocular structures, leading to corneal ulceration with loss of vision and at least one fatality (McCally et al., 1933).

Elixir of Sulfanilamide Case

In 1937, an elixir of sulfanilamide dissolved in ethylene glycol was introduced into the marketplace. One hundred seven people died as a result of ethylene glycol toxicity. The public response to these two tragedies helped prompt Congress to pass the Federal Food, Drug, and Cosmetic Act of 1938 (Pendergrast, 1984). It was this law that mandated the premarket testing of drugs for safety in experimental animals. The most compelling evidence that should be considered is "negative": since the imposition of animal testing as a result of these two cases, no similar occurrence has happened, even though society uses many more consumer products and pharmaceuticals today than during the 1930s.

Thalidomide

The use of thalidomide, a sedative-hypnotic agent, led to some 10,000 deformed children being born in Europe with only a small number of such cases in the United States (where the drug was never approved and in a time before an investigational new drug [IND] application was required for clinical trials). This in turn led directly to the 1962 revision of the Food, Drug and Cosmetic Act, requiring more stringent testing. Current testing procedures (or even those at the time in the United States, where the drug was never approved for human use) would have identified the hazard and prevented this tragedy. In fact, it has not occurred in Europe or in the United States except when

Table 1.1   Animal Models That Predicted Adverse Effects of Xenobiotics on Humans

Agent	Effect	Animal Species	In Man
Phenacetin	Neurotoxicity, carcinogenicity	Rat	Y
Thalidomide	Phocomelia	Rat	N/Y
Accutane	Developmental toxicity of CNS (neural tube defects)	Rat, rabbit, dog	Y
AZT	Bone marrow depression	Dog, rat, monkey	Y
Valproic acid	Cleft palate	Rat, mouse, rabbit	Y
Cyclosporine	Nephropathy	Rat, dog	
	Reversible immune response suppression (essential aid to organ transplantation)	Rat, monkey	
Benoxaprofen (Oraflex)	Hepatotoxicity	No	Y
	Photosensitivity	Guinea pig	
Zomepirac	Anaphylactic shock/allergy	No	Y
MPTP	Parkinsonism	Monkey	Y
Cyclophosphamide	Hemorrhagic cystitis	Rat, dog	Y
Mercury[a]	Encephalopathy	Rat, monkey	Y
Diethylene glycol[a]	Nephropathy	Rat, dog	Y
Razoxin	Myelomonocytic leukemia	Mouse	Y
Benedictin	Birth defects claimed/litogen	No	Y
Triazolam (Halcion)	Behavioral disturbances/amnesia	No	Y
Quinolones	Phototoxicity	Guinea pig, in vitro	?/Y
Temafloxacin	Hemolytic anemia	No	Y
Diazepam (Valium)	Development abnormalities	Rat	Y
Fialuridine (FIAU)	Nephrotoxicity; hepatotoxicity		Y
TGN-412	"cytokine storm"	Mouse, monkey	Y

[a] Not drugs.

the results of animal tests have been ignored. Table 1.1 presents an overview of cases where animal data predicted adverse effects in humans, and Table 1.2 provides some examples of known toxic reactions to substances in animals and humans.

Modern regulation and good science have not precluded drugs entering human use and causing predictable severe adverse effect. For example, birth defects have occurred with isotretinoin (Accutane) where developmental toxicity had been clearly established in animals and presented on labeling, but the drug has continued to be used by potentially pregnant women. An investigational biologic drug, TGN-412, demonstrated that highly potent drugs active at common receptor sites produce common extreme effects in mice, monkeys, and (at least) male humans.

Research into replacements such as cellular cultures, organs harvested from slaughterhouses, computer modeling, and physical and chemical systems has been extensive (Frazier, 1990; Gad, 2000). Although each of these has its own utility (Gad, 1989, 2000), they will not replace animals for the foreseeable future. Some degree of animal use will continue. We hope that this book will assist the responsible investigator in designing and interpreting appropriate experiments (refinement) that will require fewer animals (reduction) in which the animals are appropriately husbanded and utilized (responsibility).

The newest challenge is with protein therapeutics, which are highly humanized (and therefore difficult to select or derive an animal model that has receptors to be able to respond to the drugs' pharmacodynamic mode of action) and for which toxicity is most commonly a matter of "hyperpharmacology" at the intended receptor site and "off-target" hits at receptors at tissues other than the intended target. Here, appropriate model selection is critical.

Table 1.2 Selection of Toxic Reactions Occurring in Animals and Man

Substance	Reaction	Substance	Reaction
Acetaminophen	Hepatic necrosis	Isotretinoin, prenatal	Multiple malformations
Acrylamide	Peripheral neuropathy	Kanamycin	Cochlear toxicity
Aniline	Methemoglobinemia	Methanol	Blindness (monkey)
Asbestos	Mesothelioma	Methoxyflurane	Nephropathy (Fischer rat)
Atropine	Constipation	8-Methoxypsoralen	Phototoxicity
Benzene	Leukemia	Methyl mercury	Encephalopathy
Bleomycin	Pulmonary fibrosis	Morphine	Physical and psychological appearance
Carbon disulfide	Nervous system toxicity		
Carbon tetrachloride	Hepatic necrosis	MPTP	Parkinsonism
Cis-platinum	Nephropathy	Musk ambrette	Photosensitivity
Cobalt sulfate	Cardiomyopathy	2-Naphthylamine	Bladder cancer
Cyclophosphamide	Hemorrhagic cystitis	Neuroleptic drugs	Galactorrhea
Cyclosporin A	Nephropathy	Nitrofurantoin	Testicular damage
D&C Yellow	Eczema	Paraquat	Lung damage and fibrosis
Diethylene glycol	Nephropathy	Phenformin	Lactic acidosis
Diethylaminoethoxy-hexoestrol	Phospholipidosis of liver	Phenothiazine NP 207	Retinopathy (pigmented animals)
Doxorubicin	Cardiomyopathy	Penicillamine	Loss of taste
Emetine	ECG abnormalities	Pyridoxine	Sensory neuropathy
Ethylene glycol	Obstructive nephropathy	Scopolamine	Behavioral disturbances
Furosemide	Hypokalemia	Slow release potassium	Intestinal ulceration
Gentamycin	Nephropathy	Thalidomide, prenatal	Phocomelia (monkey, rabbit)
Hexacarbons	Peripheral neuropathy	Triothocresylphosphate	Delayed neuropathy
Hexachlorophene	Spongiform encephalopathy	Triparanol	Cataract
Isoniazid	Peripheral neuropathy	Vinyl/chloride	Angiosarcoma of the liver
Isoproterenol	Stenocardia	Vitamin A	Osteopathy
Isothiocyanates	Goiter	Vitamin D	Nephrocalcinosis

SELECTING AN ANIMAL MODEL

Choosing the appropriate animal model for a given problem is sometimes guesswork and too often a matter of convenience or logistics. One often uses a species with which one is most familiar, with little consideration as to whether the chosen species is actually the most appropriate for the problem at hand or of the model limitations. For example, the rat is probably a poor model for studying the chronic toxicity of any new nonsteroidal anti-inflammatory drug (NSAID) because the acute gastrointestinal (GI) toxicity will probably mask any other toxic effects. The guinea pig is less sensitive to most NSAIDs than the rat and would therefore be a more appropriate species for investigating the chronic (non-GI) toxicity of an NSAID, though it is still more sensitive than humans to NSAID adverse gastric effects. This practice of not rationally choosing an appropriate species for an experiment undoubtedly results in imprecise or questionable science. This alone should be considered a waste of animals and resources. It also results in additional, and sometimes duplicative, experiments. We hope that this book will contribute to the reduction and refinement of the use of animals by helping to alleviate this practice and to a better understanding of the limitations (and appropriate interpretations of findings) of specific models. The core chapters (Chapters 2 through 11) include discussions of the strengths and weaknesses of each of the common laboratory species and

recommendations for potential appropriate uses. Chapter 16 directly addresses the issue of how to select the best practical model. Chapters 12 through 15 examine the use of modern technologies and of special models.

HUSBANDRY AND CARE

The quality of an experiment often hinges on the details of animal husbandry and care. At one extreme, inappropriate handling and husbandry could result in unhealthy animals and an experiment yielding variable and irreproducible results. All animals should have optimal temperature, humidity, light cycle, light intensity, cage size and bedding, water, and dietary requirements. Rabbits, for example, have a different optimal temperature range than rats. Rats and ferrets have completely different dietary requirements. Albino rodents have very sensitive eyes, and lights of too high a candle power can cause incidental ocular damage, especially in those animals on the top row of a cage rack. Infrequent changing of indirect bedding materials can result in exposure of rodents to a high airborne concentration of ammonia, which can cause ocular damage. It is clear that ad lib feeding of rodents in chronic or carcinogenicity studies shortens their lives and alters the patterns of spontaneous tumors that occur though more recent experience suggests that the extent of these effects may not be as great as originally thought. These are all examples of how inattention to the details of animal care can compromise an experiment, particularly a long-term one. A refined and responsibly designed experiment accommodates these details. It is hoped that this book will provide a convenient source of husbandry procedures for the animal species commonly used in toxicological and pharmacological research.

Caging

Caging deserves special mention for two reasons. First, not all animals can be group housed. Hamsters, for example, are notoriously antisocial. Even breeding pairs cannot be left in the same small cage together for protracted periods. Guinea pigs, rats, and mice, on the other hand, thrive when group housed. Obviously these factors need to be considered when designing an experiment. In modern toxicology practice, animals are seldom group housed during chronic studies to maintain identification, facilitate clinical observations, and ensure necropsy of moribund or dead animals (mice, in particular, are very cannibalistic). This book will discuss appropriate housing, including instances when animals should or should not be group housed.

Second, cage size is important but the animal rights movement continues to advocate even expanding such sizes. While most investigators (and cage manufacturers) have long recognized that cages have optimal sizes, the 1989 proposed Animal Welfare Codes (which became law in 1991 and have been revised several times since) attempted to specify somewhat larger cages with several size cutoffs mediating cage changes. For example, there are three to four different cage specifications for guinea pigs depending on their age and/or weight. Many caging systems currently in use would no longer be permitted and their replacement would be very expensive. There is no scientific basis for believing that these changes will improve animal husbandry or quality of life. This is just an example of how the animal rights movement, and the resultant animal care laws, continues to influence the logistics and conduct of pharmacologists and toxicologists. This book contains in-depth discussion on current animal welfare laws (Chapter 18). The investigator needs to be aware of not only the four Rs but also of the relevant laws and regulations governing animal experimentation.

CHOOSING SPECIES AND STRAINS

It is important to pick not only the correct species for an experiment but sometimes the correct strain as well. For most of the species discussed in this book, there are a handful of commonly used strains. In some cases, an inbred strain might provide qualitative and specific characteristics that make it a good disease model such as the spontaneously hypertensive rats or obese mice. There are other more quantitative strain-related differences such as size, color, temperament, and background disease. For example, the Fischer 344 rat is smaller than the Sprague Dawley rat. The CD-1 mouse is shorter lived than the C57B6/F₁ hybrid. These differences may make a particular strain more appropriate for one experiment than others. For example, the Fischer 344 rat has a high rate of spontaneous Leydig cell tumors as compared to the Sprague Dawley rat, which would make the latter less appropriate for determining if a chemical is a testicular carcinogen. For these reasons, this book includes discussions of strain-related differences. Rats and mice provide the greatest array of strains from which to choose, including outbreed and some inbreed. There are literally hundreds to choose from, but the majority are specialized-use animals, such as the athymic nude mouse. For the majority of generalized pharmacology and toxicology testing, a relatively small handful of rat and mouse strains are used and the emphasis in those chapters will be on those more commonly used strains. Many chapters will include some mention of strain; however, the situation with dogs is somewhat different.

All domestic dogs belong to the same family, which is subdivided by breed. Only the beagle breed is purposely raised for biomedical and medical research; random sourced dogs are no longer permitted for research. Hence, the chapter on the dog will focus on the beagle. There are supplier-related differences in beagles, but these have not been systemically studied.

DOSING

To study the effects of a drug or other chemicals in an animal, the two have to be brought together, i.e., the animal has to be dosed. Dosing is the act of introducing a drug or chemical into a living organism. It requires active interaction between man and animal. There are, however, passive dosing techniques that are also used frequently in which the chemical is placed in the animal's air, water, or feed, and the animal doses itself by breathing, drinking, or eating. Hence, administering an antibiotic intravenously is active dosing; giving it in the feed is passive dosing. In the former case, dosimetry (i.e., calculating milligrams per kilograms of exposure) is generally intuitively simple (an exception being for the dermal route). In the latter case, other measurements must be taken (e.g., feed consumption) and a variety of formulas are used in dosimetry. The main routes used for active dosing are oral, intravenous (IV), intraperitoneal (IP), dermal, and subcutaneous (SC). Other routes are sometimes used, and these will be mentioned where appropriate (for a complete discussion of different routes, see Gad and Chengelis, 1998, Chapter 10, and Gad, 2000, 2009). For oral dosing, for example, one may have a choice of using capsules or gavaging. However, capsules are rarely used with rats, and gavage is seldom used with dogs. When necessary, a dog can be gavaged, but the technique is different from that used with rats. IV dosing of ferrets is especially difficult, but can be done. It is hoped that this book will present the appropriate techniques, "tricks of the trade," so that animals can be appropriately and humanely dosed.

Second, some of the information (e.g., average feed consumption) and formulas needed to calculate or estimate dosimetry in passive dosing procedures are presented and discussed. With regard to dosing and dosimetry, it should be kept in mind that the terms "dose" and "dosage" are not synonymous. The dose is the total amount of test article given, e.g., 1000 mg. The dosage is a rate term and is the dose divided by the weight of the test animal, i.e., 1000 mg/10 kg (for a dog) = 100 mg/kg.

For some agents (particularly oncology drugs), this is presented in terms of quantity per meter squared (m^2) of body surface area. In most instances, when one speaks of a dose–response curve, a dosage–response curve is being described.

ANIMAL PHYSIOLOGY

All animal species and strains have their own distinctive physiology. As a result, values pertaining to blood pressure, breathing rates, ECGs, rectal temperatures, and normal clinical laboratory parameters often vary between species. Clearly, appropriate interpretation of an in vivo experiment requires one to have a firm understanding of these baseline data. For example, there are well-established differences between species with regard to red blood cell size. What is normal for a dog would be high for a rat. The converse is true for breathing rates. This book provides a convenient source for these important background data and abilities to perform metabolism develop with age, and may not be present in the neonate or juveniles (Klinger, 1982).

Background Incidence of Disease and Neoplasia

All animals also have their own baseline, or natural incidence, or diseases that complicate the conduct and interpretation of chronic toxicity experiments. The background incidence of liver tumors in C57B6/F$_1$ mice is quite high. It would, perhaps, be prudent to investigate a suspect hepatocarcinogen in a species with a lower spontaneous incidence than these. Ferrets in the United States are currently contaminated by the Aleutian mink virus, which could make this species inappropriate for chronic experiments. The background incidence of these diseases and pathological lesions are discussed to aid the investigator in choosing the more appropriate species for an experiment and in the interpretation of the results.

Responses to Biologically Active Agents

An animal's responses to drugs or other biologically active agents might be just as important as the background incidence of disease, and species-related differences in sensitivity are important for two reasons. First, animals will often have to be anesthetized or receive other treatment such as antibiotics during an experiment. Appropriate dosages vary between species. Thus, this book presents the appropriate dosages of common anesthetics for the model species discussed here.

Second, the other reason species-related differences are important is that in toxicity testing, these differences are the major hurdle in applying toxicity data to human hazard assessment. This is perhaps too broad a topic for a single book, but mention is made so that an investigator is aware of such differences. Cats, for example, are far more sensitive to digitoxin ($LD_{50} \cong 180$ μg/kg po) than other species, such as the rat ($LD_{50} \cong 56$ mg/kg po, as reported by the National Institute of Occupational Safety and Health, 1980).

There can also be qualitative differences between species. Morphine, for example, is infamous for causing different clinical signs in different species: straub tail in mice, catatonia in rats, and extreme reactivity in cats. Some of the more frequent examples of these distinctions will be mentioned in the core chapters. The salient message is that species often differ both quantitatively and qualitatively in their responses to drugs and/or chemicals. These differences must be investigated and considered in choosing a species for an experiment and in interpreting the results. Incidentally, cats (with the exception of veterinary products intended for use in cats) are seldom used in toxicity testing and are used in pharmacology mainly for acute, terminal, neurophysiological experiments. For these reasons, an in-depth discussion of cats is not included in this book.

Absorption, Distribution, Metabolism, and Excretion of Xenobiotics

When studying the effects of drugs and other chemicals on intact animals, it is also vitally important to investigate the processes of absorption, distribution, metabolism, and excretion (ADME). These have been intensely and widely studied. Space does not permit a review of this large body of work. Some basic degree of knowledge must be presumed. We have compiled a list of references to which the reader can refer if additional information is needed (Table 1.3). For the remainder of this chapter, we will touch upon some basic principles that apply across all species. In each individual core chapter (Chapters 2 through 10) of this book, some basic information on ADME will be presented on a species-specific basis. The emphasis will be on providing the information necessary to assist one in (a) the appropriate selection of an animal model, (b) the design of the experiment, (c) the interpretation of resultant data, and (d) the applicability of the results to humans.

The principles that govern absorption and distribution apply fairly equally across all species (Cheeke and Dierenfeld, 2010; Pratt and Taylor, 1990; Washington et al., 2001), and therefore will not be discussed to any great extent on an animal-by-animal basis. It is most difficult to predict species differences in bioavailability (absorption across GI tract into the blood) or systemic bioavailability (bioavailability + first-pass metabolism) of a specific chemical. Species differences in gastric

Table 1.3 Summary of General Reviews of Xenobiotic Metabolism

Topic	Source
General reviews on process of drug metabolism and disposition	LaDu et al. (1979)
	Goldstein et al. (1974)
	Klaassen (2008)
	Parkinson and Ogilvie (2008)
	Kemper et al. (2014)
	Rozman (1988)
	Levy et al. (2000)
Cytochrome P-450	Gonzales (1988)
	Black and Coon (1986)
	Kadlubar and Hammons (1987)
	Lewis (2001)
Flavin-dependent microsomal mixed function oxidase	Ziegler (1988)
	Tynes and Hodgson (1985)
Epoxide hydrolase	Seidegard and DePierre (1983)
	Oesch (1972)
Glutathione S-transferase	Jarina and Bend (1977)
	Pickett and Lu (1989)
UDP-glucuronosyl transferase/glucuronidation	Boutin (1984, 1987)
	Mulder et al. (1986)
	Siest et al. (1989)
PAPS-sulfotransferase/sulfate formation	Singer (1985)
	Jacoby et al. (1984)
Amino acid conjugations	Hirom et al. (1977)
Acetylations	Lower and Bryan (1973)
Esterases	Leinweber (1987)
Alcohol metabolism	Hawkins and Kalant (1972)
	Crabb et al. (1987)
Biliary excretion	Klaassen and Watkins (1984)
	Levine (1978)

or intestinal pH, for example, may dictate species differences in GI permeability to specific chemicals, but will not account for differences in GI transit time or hepatic metabolism. Assumptions based solely on phylogenetic grounds can be quite misleading. We had recent experience with a drug found to be bioavailable in the rat and dog, but not at all absorbed in the monkey. In fact, the dog was the species most similar to the human. One needs to strive to ascertain test article bioavailability experimentally for any specific chemical, as general principles always come encumbered with exceptions.

Absorption

After dosing, a chemical must be absorbed and distributed to receptor sites in order to cause an effect. Absorption is the process of the chemical passing through a barrier to gain access to the general systemic circulation. The most common dosage routes are oral, inhalation, topical, IP, IV, SC, and intramuscular (IM). Absorption is not generally a problem by the latter three routes as the test substance is introduced directly to the body. It is normally a foregone conclusion that drugs so administered will reach the systemic circulation. Plasma concentrations will depend on rates of delivery (IV) or rates of diffusion (IM/SC). Although there are some technical concerns, the principles are either independent of species, or the species differences are obvious. For example, because of relative small muscle mass and rapid circulation time, drugs given intramuscularly will more rapidly equilibrate in rats than in monkeys. Via the IP route, systemic availability will depend not only on the rates of diffusion but also on the first-pass metabolism effect. There are no known species differences with regard to IP absorption, but there are species differences with regard to rates of hepatic metabolism that may dictate the degree of first-pass metabolism. Interestingly, first-pass effects are generally of greater concern in smaller species, rat and mice, where the IP route is more commonly used.

With regard to the oral, dermal, and inhalation routes, there are very real species differences. For example, thickness and length of the small intestine, size of cecum (if indeed there is one), and gut transit time will all play a role in GI absorption.

Species differences in facilitated or active transport may also play a role in absorption. Whether an animal is an obligate nose breather or not, the structure of the nasal turbinates, respiration rate, and minute volume will all influence the size and number of the particles reaching the alveoli by the inhalation route. The rat is a poor model for inhalation pharmacokinetic studies in extrapolating the results to humans for these reasons. There are well-described differences in skin structure that control dermal absorption and result in species differences. Such species differences will vary with chemical class.

This book may help one sort through this maze, but there are few scientifically sound generalizations. Our best recommendation is that investigators substantiate their assumptions on dermal or inhalation absorption before rendering any conclusion on studies conducted using these routes of administration.

Distribution

After gaining access to the systemic circulation, the toxin/drug is distributed among the organs. Distribution will depend on

- Blood flow to the organ
- Extent and avidity of binding to plasma proteins
- The "natural" affinity a particular organ may have
- The degree and extent to which the chemical crosses barriers such as the blood–brain barrier, the placenta
- The extent to which clearance (metabolism and/or excretion) competes with these processes
- Partitioning based on lipophilicity and hydrophilicity

There are probably species differences with regard to all these processes. Not all have been vigorously explored, however. For example, there are few comparative studies on the blood–testis barrier or comparisons on plasma protein binding of different chemicals in the monkey, so the database in this area is surprisingly small. A few transspecies comparisons of plasma protein binding have been done. As a broad generalization, binding is most extensive in humans and least extensive in mouse. Such information is presented and discussed in the core chapters, but the reader should be aware of the holes in the available knowledge.

Metabolism

In the area of ADME, the processes of metabolism or bioconversion are of greatest concern with regard to species-specific differences. Indeed, species differences in metabolism are a leading cause for species differences in toxicity. First, very few administered xenobiotics are excreted unchanged. Therefore, rates of metabolism often dictate the time length of a pharmacodynamic response. Second, metabolism of a xenobiotic may result in metabolites of similar potency and/or produce metabolites that are responsible for toxicity. For example, most genotoxic carcinogens require metabolic activation. Finally, because the metabolism of xenobiotics is an enzyme-based phenomenon, it shows a great deal of species differences. For example, Williams (1972) examined the metabolism of phenol, a relatively simple chemical, in 13 different species and found that no 2 species provided the same spectrum of metabolites. Species differences can be either quantitative (differing amounts of the same metabolites) or qualitative (different metabolites). Because of the importance of metabolism in toxicity testing, each individual animal chapter contains in-depth discussion of xenobiotic metabolism.

Xenobiotic Metabolism

The area of species differences in xenobiotic metabolism is not new. Some of the efforts in this area are summarized in Table 1.3. It is not the objective of this book to provide yet another interspecies comparison, but rather to present information on a species-specific basis. For example, what type of regimen is required to induce increases in microsomal multifunction oxidase (MMFO) activity in the dog? The metabolism of xenobiotics by mammals is a phenomenon that has been recognized since 1842 when Keller (Mandell, 1972) identified that benzoic acid was excreted in the urine as the 1 cine conjugate (hi uric acid). As a modern science, drug (or xenobiotic) metabolism was formalized in the late 1940s when Williams (1947) (Caldwell, 1981) published the first text on the subject. Williams (see Williams, 1974 for references and Williams, 1979 for reviews) has been particularly instrumental in the area of species differences in metabolism. Early works in this area tended to concentrate on isolating and identifying various conjugations of simple chemicals given to intact animals. Miller and Mueller published on the importance of liver microsomes in xenobiotic metabolism in their studies on the oxidative metabolism of aminazodyes (Mannering, 1972; Mueller and Miller, 1949). The field has grown explosively since the mid-1950s, catalyzed by the studies of Brodie and colleagues in the United States (Brodie et al., 1955; Quinn et al., 1958) and Remmer in Germany (Mannering, 1972; Remmer and Merker, 1963). Their works confirmed the quantitative importance of the liver in xenobiotic metabolism, and the major underlying enzymes were located in the microsomal fraction. It was during this period that the practice of naming an enzyme by its activity, such as aminopyrine demethylase or aniline hydroxylase, was adopted. It was only later that it was recognized that all these activities are catalyzed by the same enzymes (or family thereof), i.e., the cytochrome P-450-dependent microsomal mixed function oxidase system (Gonzales, 1988; Guengerich, 1988). Cytochrome P-450 was discovered almost a decade after Miller and Mueller described microsomal metabolism requiring NADPH (Coon, 1978; Coon and Vaz, 1987; Klingenberg, 1958; Mannering, 1972). The importance of the identification and characterization of the cytochrome P-450-dependent MMFO system to the

fields of biochemistry, pharmacology, and toxicology could not be understated. The reader is referred to any one of several reviews of the system (see Tables 1.1 and 1.4).

The process of xenobiotic metabolism has traditionally been divided into phase I (oxidative) and phase II steps. In general (as reviewed more extensively by Kemper et al., 2014 and Lehman-McKeename, 2008), all mammalian processes are designed to convert lipophilic chemicals to more polar and more easily excreted metabolites. In reality, the process can be more complicated than two steps because the products of phase I oxidation can be (1) further hydroxylated at different sites, (2) further oxidized at the same site (by a different enzyme such as alcohol dehydrogenase), or (3) conjugated with glutathione or glucuronic acid, sulfate, or one of several amino acids. This process is discussed in greater detail elsewhere (see Tables 1.1 and 1.2). The result is that any one xenobiotic may have an astonishing spectrum of metabolites. For example, benzene is a relatively simple chemical, yet over 15 different metabolites have been described. There is in general, in mammals, a homology between species upto P-450 isozymes, though varying degrees of temperature induced antibodies may cause differences (Miura et al., 1989).

Enzymes Involved in Xenobiotic Metabolism

The main enzymes involved in xenobiotic metabolism are fairly uniform across species. In all mammalian species, the liver is quantitatively the most important site of xenobiotic metabolism, and the MMFO system is the most important enzyme. While this system is ubiquitous, there are species differences in isozymic characteristics, substrate specificity, activity, and inducibility. More recently, Davin-dependent MMFO, which is distinct from the MMFO, has been identified (Ziegler, 1988) and has been shown to play a role in the metabolism of many chemicals. There are also differences in phase II enzyme activities and cosubstrate availability (Gregus et al., 1983). Seldom do two species dispose of the same chemical that way. Each species produces a spectrum of metabolite or chomatographic "fingerprints" that are often distinct. The characteristics of the MMFO for each of the most highly used species will be discussed in detail in this book. Other enzyme systems such as the flavin-dependent (noncytochrome P-450) monoxygenase may also be involved in xenobiotic oxidative metabolism and are discussed where available information permits. The species characteristics of other important enzymes such as epoxide hydrolase and UDP-glucuronosyl transferase are also discussed. Some enzymes are ubiquitous, such as the alcohol dehydrogenase and carboxylesterase. All species metabolize primary alcohols to aldehyde and subsequently to carboxylic acids. This will only be discussed, therefore, when there is some species-specific characteristic. This is also true for esterases, as all species rapidly hydrolyze esters.

Excretion

The processes of elimination will not be dealt with in great detail in the core chapter. This is not to say, however, that excretion is not important. Like absorption, elimination can be both active and passive, and most xenobiotics are passively excreted. In most cases, conjugated metabolites are actively excreted. The process of xenobiotic metabolism can be viewed, to a certain extent, as packaging for the excretory process. Across species, the active excretion of a metabolite by the liver into the bile is probably (quantitatively) the most important active excretory process concerning xenobiotic disposition. Glucuronide conjugates (as reviewed by Klassen and Watkins, 1984; Levine, 1978; and Williams, 1972) are actively excreted by the liver into the bile ultimately into the feces. Amino acid conjugates, in contrast, tend to be excreted by the kidney into the urine. These are definite species-related differences in the molecular weight cutoff between 300 and 500 for the biliary transport of the metabolite that dictate whether a metabolite will end up in either the feces or the urine.

Species differences do not follow any particular phylogenetic lines. For example, rats and dogs effectively excrete phenolphthalein glucuronide (50% in bile), whereas guinea pigs and monkeys do not (<10%) (Williams, 1972). While there are species differences in excretions, these tend to

Table 1.4 Compilation of Selected Papers that Compare Xenobiotic Metabolism in Different Species

Species Compared	Parameters Examined	Comments	References
Dog, guinea pig, rat, rabbit, monkey, human, mouse	Gastrointestinal differences that affect absorption; plasma and tissue binding; drug metabolism in liver and intestine	Rhesus monkey best predictor for ADME in man. Excellent bibliography.	Rozman (1988)
Rat, mouse, guinea pig	GSH-T	With CDNB total activity mouse > guinea pig > rat. Parallel to AFT sensitivity. Quantitative differences in isoenzymes.	Neal et al. (1987)
Rat, hamster, mouse, guinea pig	Induction of P-450 and MMFO activities (AP demethyl, BP-OH, EC-deethyl) by 2-AAF and 3-MC	In general, MMFO activity, rat had the lowest, but BP activity the most inducible. In some species, induction had no effect or decreased some activities.	Astrom et al. (1986)
Mouse, guinea pig, rabbit, hamster	GSH-T (1-chloro-2,4-dinitrobenzene)	Hamster > rabbit = guinea pig > mouse > rat. S-sepharose elution patterns different.	Igarashi et al. (1986)
Rat, rabbit, dog, mouse	Chlorfenvinphos deethylation (in vitro)	Dog > rabbit = mouse > rat with same order to LD_{50}.	Hutson and Logan (1986)
Rat, dog, monkey	Metabolism and kinetics of tolrestat	Highest bioavailability in rats. More unchanged drug in dogs and monkeys.	Cayen et al. (1985)
Rat, mouse, guinea pig	Induction of MMFO, GSH-T, EH	EH-M guinea pig > rat > mouse, but guinea pig is less inducible. For GSH-T, mouse > rat > guinea pig and guinea pig not induced. For MMFO, guinea pig > mouse > rat.	Thabrew and Emerole (1983)
Rat, dog, monkey	Inducing effect of hexahydroindazole (P-450, AP demethyl, AN-OH)	Increases in relative liver weights in all species. For P-450, monkey > rat > dog; for AP, monkey > rat = dog; for AN, rat = monkey > dog (gram basis, different if on protein). Best induction in dog.	Lan et al. (1983)
Rat, mouse, guinea pig, dog, monkey	Conjugation reactions	A review.	Caldwell (1982)
Rats, rabbits, hamster, guinea pig, ferret	Metabolism of glyceryl trinitrite	Species differences in plasma half-life a function of body weight.	Ioannides et al. (1982)
Various: emphasis on dog, mouse, rat, rabbit, monkey	Various aspects of metabolism covered: spectrum of metabolites, plasma half-lives, developmental differences, inhibitors, inducers	Excellent comprehensive review with emphasis on mixed function oxidase activity.	Kato (1979)
Rat, mouse, rabbit, hamster, guinea pig	Changes in iron "spin state" as determined by EPR induced by different binding spectra	Proportion of high spin P-450 in vivo: Rabbit > guinea pig = hamster = mouse > rat.	Kumaki et al. (1978)

(*Continued*)

Table 1.4 (*Continued*)   Compilation of Selected Papers that Compare Xenobiotic Metabolism in Different Species

Species Compared	Parameters Examined	Comments	References
Rat, rabbit, guinea pig	Different inducing agents	Glucuronide formed only by liver and other organs involved in sulfation. Both produced in liver of rat, rabbit and guinea pig; only sulfate formed by mouse.	Wong (1976)
Rat, mouse, rabbit, hamster, guinea pig	Protein, P-450 content, reductase concentrations, model substrates, GSH and UDPG transferase activities, lung, liver, kidney	For all species, liver most active with lung and kidney 15%–40% of liver. No species superior in all activities, but hamster tended to have greatest activities.	Litterst et al. (1975)
Rat, mouse, guinea pig, rabbit	Microsomal protein, BP-OH, UDPG transferase, small intestine versus liver	No real differences in microsomal protein (30–35 mg/g). For BP, liver > gut for all species. For liver guinea pig = mouse > rat > rabbit; for gut, guinea pig > rabbit > rat = mouse; for UDPG liver, guinea pig > rabbit = mouse > rat; for UDPG-gut, rabbit > rat > mouse = guinea pig.	Haietanen and Vainio (1973)
Rat, mouse, guinea pig, hamster, rabbit, dog, pig, monkey	Metabolism of [³H]styrene oxide: EH and GSH-T in liver, lung, and kidney	For EH, liver > kidney > lung. In general, mouse is lowest and primate is highest. For GSHT, liver > kidney > lung. GP is highest and primate is lowest. Includes lit comp. of EH activities.	Pacifici et al. (1981)
Rat, mouse, guinea pig, rabbit, pig, monkey	P-450 b5, cytochrome c reduced, K_m and V_{max} of various substrates	Not big differences in micro: P-450 ranges from 0.38 (pig) to 0.75 nmol/mg (guinea pig). b5 ranged from 0.20 (mouse) to 0.49 nmol/mg (guinea pig). Cytochrome c red ranged from 115 (rabbit) to 136 (guinea pig) nmol/min/mg.	Amri et al. (1986)
Rat, mouse, guinea pig, hamster	Induction of EH UDPG transferase and GSH-T by 2-AAF or 3-MC	Large species variation (3–12-fold) in control activities. Except for EH in guinea pig, enzymes induced only in rat by 2-AAF. Except for GSH-T in hamster, enzymes induced only in rat by 3-MC. Rats not representative of activities or inducibility of other species.	Astrom et al. (1987)
Rat, mouse, guinea pig, hamster	Effects of DDT on AHH activity and cytochrome P-450	Induction in hamsters, decreases in other species. Acute toxic effects depend on route and species.	Haietanen and Vainio (1976)

(*Continued*)

Table 1.4 (*Continued*)   Compilation of Selected Papers that Compare Xenobiotic Metabolism in Different Species

Species Compared	Parameters Examined	Comments	References
Rat, mouse, guinea pig, hamster	Total GSH, γ-GTP, GSH synthetases, peroxidase, and reductase	GSH lowest in guinea pig, highest in mouse. Synthesis lowest in hamster, highest in rat. γ-GT much higher in guinea pig. GSH-T highest in guinea pig, lowest in rat, and not affected by fasting. GR lowest in rat = mouse, highest in hamster; fasting affects species dependent. Px highest in mouse = hamster, lowest in rat and mouse, depend on substrate and fasting state.	Igarashi et al. 1983
Rat, mouse, rabbit, guinea pig	GSH-T activity with different substrates, different age animals, and with different inducing agents	Wide range of activities, depending on species and substrate. Age-related changes evident in all species; peak (up to 120 days) varies with species. Activity inducible in all species, but extent depends on inducer and substrate. Rat mouse most inducible.	Gregus et al. (1985)
Rat, mouse, hamster, guinea pig, monkey	Review on role of intestinal microflora in drug metabolism. Excellent bibliography with references to primary articles and other reviews	Gut flora (β-glucosidase and β-glucuronidase; nitro, nitrite, and azo reductases) have large species difference; guinea pigs tend to have lowest amounts and mice the highest.	Rowland (1988), Rowland et al. (1986)
Rat, mouse, hamster, rabbit, guinea pig, dog, primate, others	Excellent review. Qualitative differences (lack of specific enzymes) and quantitative differences. Emphasis on in vivo data. Species differences in the metabolism of [1–14c-acetyl]phenacetin	Examples: Only in the rat is aromatic hydroxylation the major route of amphetamine metabolism. Dogs and guinea pigs have a defect in N-acetylation. Guinea pigs do not make mercapturic acids.	Williams (1972, 1979)
Rat, rabbit, guinea pig, ferret	Case reviews on how species differences in drug metabolism lead to differences in toxicity	Deacetylation highest in rat and ferret, aromatic hydroxylation high in ferret, low in others. Glucuronide formation dominant in rabbit, guinea pig, ferret: sulfation is dominant in rat.	Smith and Timbrell (1974)
Rat, mouse, guinea pig, hamster, rabbits, dogs, primates	Review chapter in monograph. Species differences in biotransformation, plasma protein binding, biliary excretion, and pharmacokinetics	Example: Dog has increased risk of bladder cancer (in response to) aromatic amines because of ability to N-hydroxylate but limited capacity to acetylate.	Calabrese (1988)

(Continued)

Table 1.4 (*Continued*)   Compilation of Selected Papers that Compare Xenobiotic Metabolism in Different Species

Species Compared	Parameters Examined	Comments	References
Rat, mouse, guinea pig, hamster, rabbits, dogs, primates		Example: In general, protein binding is highest in primate and lowest in mouse.	Cayen (1987)
Rat, mouse, rabbit, guinea pig, dog	A citation classic. Species and other factors, in the metabolism of four different chemicals explored	In general, most rapid half-life in mice, longest in dogs. Correlates with rates of microsomal demethylation.	Guinn et al. (1958)
Rat, mouse, guinea pig, rabbit, rat, dog, quail, trout	Cytochrome P-450, MMFO, EH, UDPG transferase, PAPS-sulfotransferase, *N*-acetyl transferase	A good basic comparison. Convenient source for species comparison.	Gregus et al. (1983)
Rat, mouse, guinea pig, rabbit, dog	Drug metabolism by nasal tissue in vitro	Highest activities in hamster, lowest in dog.	Hadley and Dahl (1983)

Abbreviations: EH, epoxide hydrolase; UDPG, UDP-glucuronic acid.

be overshadowed by the species differences in metabolism. That is, a particular species may not need an efficient biliary excretory process because with a particular chemical, it may produce glucuronides sparingly or not at all. This information may be of interest to the pharmacokineticist in determining where to look for a metabolite. Generally, however, such information is of academic interest to the pharmacologist or toxicologist in interpreting an experiment because glucuronides are generally inactive end products, and it does not really matter whether they end up in the urine or feces. (As with any rule, there are exceptions: Mulder, 1986, cites several examples of glucuronides being active metabolites, i.e., causing toxicity.) There are several instances, however, where biliary excretion actually influences the toxicity of a chemical, such as with some of the cardiac glycosides or heavy metals where biliary excretion occurs without metabolism and biliary excretion is the "detoxification" mechanism (Klassen and Watkins, 1984). Cayen (1987) has pointed out that species-related differences in indomethacin-induced intestinal damage directly correlate to the degree of exposure of the mucosa owing to biliary excretion and resultant enterohepatic circulation. Such instances are discussed on a species-specific basis where such data permit.

SUMMARY

This new edition is a revised attempt to provide a source book of basic information on laboratory animals for safety assessment, incorporating the new technology that has become available. I trust that this book will provide a convenient source of information for either the skilled or novice investigator to aid in the design and interpretation of in vivo pharmacological or toxicological studies.

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The Mouse

Shayne Cox Gad, Charlie Clifford, and Dawn Goodman

CONTENTS

TOXICOLOGY

Shayne Cox Gad

History

The domesticated mouse of North America and Europe (*Mus musculus*) is the most widely used animal in medical research. The mouse is a member of the order Rodentia, family Muridae, and subfamily Murinae.

The use of the mouse in biomedical research has been chronicled for several hundred years. William Harvey (1578–1657) published the results of his work on animal reproduction and blood circulation based in part on his work with mice (Harvey, 1616, cited in Morse, 1981). Joseph Priestly used mice in exploring the phlogiston theory (Priestly, 1775), and Antoine Lavoisier used mice in his studies of the physiology of respiration (Lavoisier, 1777) (both cited in Morse, 1981).

Mice were selectively bred for coat color for many centuries, but in the early 1900s, efforts turned to breeding strains of mice that might mimic human disease states. Subsequently, inbred strains were derived that were particularly susceptible or resistant to various types of cancers and viruses. A strain is considered to be inbred when it has been derived by brother X sister matings for 20 or more consecutive generations (F20) and can be traced to a single ancestral breeding pair in the 20th or subsequent generations. Certain other breeding systems (e.g., parent X offspring) may be substituted as long as the inbreeding coefficient achieved is at least equal to that at F20 (Lyon, 1981). The genetic groundwork was laid for most of the strains of inbred mice currently in use by researchers such as William E. Castle, Clarence C. Little, and Leonell C. Strong during the period of about 1900–1930 (Morse, 1981).

While highly inbred strains have proven invaluable in fields such as genetics and histocompatibility research, a school of thought developed that random bred or specifically outbred strains might more closely represent man in many areas of medical research. Such random breeding programs attempt to achieve a level of genetic variability similar to the initial (noninbred) population and, in so doing, preserve the "hybrid vigor" associated with heterozygosity.

Many of the inbred and outbred strains of mice currently in use are referred to as "Swiss" strains. All of these Swiss strains are traceable to a group of two male and seven female albino mice obtained from the noninbred stock of Dr. A. de Coulon of Lousanne, Switzerland and imported into the United States by Dr. Clara Lynch of the Rockefeller Institute in 1926 for use in cancer research (Lynch, 1969, cited in Hill, 1983).

Mouse in Toxicological Research

As discussed in Chapter 1, the choice of a species for toxicity testing is based on consideration of a range of variables. Ideally, if toxicity testing is intended to provide information on the safety of a test article in or by humans, the species chosen for testing should be most similar to the human in the way it handles the test article pharmacodynamically. Substantial differences in absorption, distribution, metabolism, or elimination between test species and the target species, e.g., the human, will reduce the predictive value of the test results. From a practical standpoint, often the pharmacokinetics are unknown in humans or the variety of available test species at the time of species selection. For this reason, testing is usually conducted in at least two species. Generally, one of those species is usually a rodent and the other a nonrodent. The two most commonly used rodent species are mice and rats, and often toxicity testing is conducted in both of those species.

Mice have many advantages as test animals for toxicity testing. They are small, relatively economical to obtain, house, and care for, and they are generally easy to handle. Mice are generally more economical than rats in these respects. While mice may attempt to escape or bite handlers, with regular, gentle handling, they are easily managed. Other advantages of the species include a short gestation period and a short natural life span. These characteristics allow studies that include evaluation of reproductive performance or exposure to a test article for periods approaching the expected life span (e.g., evaluation of carcinogenic potential) to be conducted in a practical time frame. High-quality healthy mice are available from reliable commercial suppliers. Many genetically well-defined highly inbred, specifically or randomly outbred strains are available. Mice have been used in biomedical research for hundreds of years, and because of this, many technical procedures have been developed for use with the species, and a vast body of historical data are available for most strains. This historical database includes information on optimal nutritional and housing requirements in addition to data such as the expected background incidence of various diseases and types of tumors in untreated animals and is continuously being added to (Blackwell et al., 1995).

There are also disadvantages to using mice, and most are related to the small size of the animal and limits that this imposes. The smaller size and higher metabolic rate compared to the rat renders the species a bit less hearty than rats. Deviations in environmental conditions such as an air conditioning failure or failure in an automatic watering system typically have more severe effects on the smaller species such as mice than the same deviations have on rats. Owing to their high level of natural activity, most strains of mice will not become as docile or easy to handle as rats that have received equivalent handling. Small size often precludes or renders more difficult a number of procedures that are commonly conducted in toxicity testing, such as the collection of large samples or repeated samples of blood and urine, electrocardiographic evaluation, and some necropsy evaluations. The Food and Drug Administration (FDA) provides human equivalent dose interspecies conversion factors for converting most animal model no observed adverse effect levels (NOAELs) or such to a human equivalent. The conversion factor for the mouse is 12.1, meaning an observed NOAEL in a mouse of 12.1 mg/kg/day would be converted to 1.00 mg/kg/day for conservative human administration. This relatively high value imposes a *de facto* penalty on using mice as one of the species in a safety assessment.

This section will provide brief summaries of some of the normal physiological values and salient features of the species and some of the specific strains that may be useful in selecting an appropriate species and strain for toxicity testing.

Normal Physiological Values

Selected normal physiological values for mice are shown in Tables 2.1 and 2.2. Median survival of a number of groups of Charles River CD-I outbred mice is shown in Table 2.3.

These normal values will vary depending upon the strain of mouse, supplier, condition at arrival, type of feed, environmental and housing conditions, and in some cases, time of year. These data should be considered as a reference, but will not necessarily represent experience in any particular laboratory.

Species Differences

Mice are similar to other common laboratory animal species and to humans in many ways, yet the differences should not be underestimated. Mice have a high metabolic rate compared to other species. This fact alone may result in increased or decreased toxicity of a test article, depending on the specific mechanism of intoxication. In many cases, high metabolic rate may be associated with rapid absorption, distribution, metabolism, and elimination of a test article. Mice are obligate

Table 2.1   Normal Physiological Values

General	
Life span	
Average	1–3 years
Maximum reported	4 years
Adult weight	
Male	20–40 g
Female	18–40 g
Surface area	0.03–0.06 cm^2
Chromosome number (diploid)	40
Food consumption	4–5 g/day
Water consumption	5–8 mL/day *ad libitum*
Body temperature	36.5°C
Oxygen consumption	1.69 mL/g/h
Reproductive	
Age, sexual maturity	
Male	50 days (20–35 g)
Female	50–60 days (20–30 g)
Breeding season	Continuous, cyclic
Estrus cycle	4–5 days
Gestation period	
Average	19 days
Range	17–21 days
Litter size	
Average	12
Range	1–23
Birth weight	1.5 g
Age begin dry food	10 days
Age at weaning	16–21 days (10–12 g)

Sources: Data derived from Jacoby, R. O. and Fox, J. G., Biology and diseases of mice, in *Laboratory Animal Medicine*, Fox, J. G., Cohen, B. J., and Loew, F. M., eds. Academic Press, New York, 1984, pp. 31–89; Purina Mill, Inc., *Animal Diet Reference Guide*, Indianapolis, IN.

Table 2.2   Normal Physiological Values

Cardiovascular	
Heart rate	
Average	600/min
Range	320–800/min
Blood pressure	
Systolic	133–160 mm Hg
Diastolic	102–110 mm Hg
Blood volume	
Plasma	45 mL/kg
Whole	78 mL/kg
Hematocrit	41.5%
RBC life span	20–30 days
RBC diameter	6.6 μm
Plasma pH	7.2–7.4
Respiratory	
Rate	
Average	163/min
Range	84–230/min
Tidal volume	
Average	0.18 mL
Range	0.09–0.38 mL
Minute volume	
Average	24 mL/min
Range	11–36 mL/min

Sources: Data derived from Jacoby, R. O. and Fox, J. G., Biology and diseases of mice, in *Laboratory Animal Medicine*, Fox, J. G., Cohen, B. J., and Loew, F. M., eds. Academic Press, New York, 1984, pp. 31–89; Purina Mill, Inc., *Animal Diet Reference Guide*, Indianapolis, IN; Lang, P. L., Survival of CrI:CD-I BR mice during chronic toxicology studies. *Charles River Laboratories Reference Paper*, Wilmington, MA, 1989.

Note: Data represent median survival of Charles River CD-I outbred albino mice enrolled in 24-month chronic toxicity studies at pharmaceutical or contract toxicology laboratories.

Table 2.3   Median Survival Rates of 16 Groups of Control Mice (%)

	Period of Time on Study (Months)			
Sex	**6**	**12**	**18**	**21**
Male	98%	91%	63%	46%
Female	98%	95%	74%	68%

nose breathers and have more convoluted nasal passages than humans. This may result in an excess of respirable test article deposited in the nasal passages, resulting in either increased or decreased relative toxicity, depending upon the most critical site of absorption. The small size of the mouse compared to other common laboratory species offers a significant advantage if the test article is expensive or in short supply.

As an approximation, a mouse weighs about 10% as much as a rat, about 5% as much as a guinea pig, about 1% as much as a rabbit, and less than 1% as much as a dog or primate. Material requirements to administer equivalent dose levels are usually proportional to body weight, so the test article savings associated with the mouse are evident. The small size of a mouse results in high

surface-area-to-body-mass ratio, which in turn causes the mouse to be relatively intolerant of thermal and water balance stresses. The kidneys of a mouse have about twice the glomerular filtering surface per gram of body weight as a rat, and owing to the specific architecture of the murine kidney, they are capable of producing urine that is about four times as concentrated as the highest attainable human concentrations (Jacoby and Fox, 1984). These characteristics of renal architecture and function may be important to the toxicity of some test articles. Mice differ from most species by the formation of a persistent vaginal plug after mating. The presence of a vaginal plug is easily detected, is considered evidence of mating, and is a useful characteristic during the conduct of reproductive studies.

It is also frequently the case in pharmaceutical research and development that the nonclinical efficacy model for a new drug is in the mouse, making it the natural choice for rodent evaluation of the drug.

Strain Differences

In addition to differences between mice and other species, there are important differences among different strains of mice. The appropriate choice of a strain of mice for a particular toxicity study should consider the specific objectives of the study and the specific characteristics of candidate strains that might assist or hinder in achieving those study objectives.

One difference among strains is in the normal body weights of various strains at different ages. These differences are summarized for selected strains available from the Charles River Breeding Laboratories in Table 2.4. Outbred strains tend to be larger at maturity than inbred strains, with the CD-I strain reaching the highest mean weights at 56 days of age of those strains in Table 2.4. The CF1 strain has been reported to be highly resistant to mouse typhoid and to be relatively resistant to salmonellosis (Hill, 1981). Nude or athymic strains of mice are more sensitive to tumor development than heterozygous strains. These sensitive strains develop the same types of tumors as those seen in more conventional strains, but the incidences are higher and the latency periods shorter. There is a wide spectrum of susceptibility to spontaneous lung tumors in various strains of mice, and evidence suggests that there is a high correlation between spontaneous incidence and chemical inducibility in those various strains (Shimikin and Stoner, 1975).

The inbred strain A mouse appears to be the most susceptible to lung tumors and forms the basis of a lung tumor bioassay, with tumors inducible within 8 weeks or less of treatment. Susceptibility of various strains to the initiation and/or promotion of skin tumors has also been shown to differ greatly (Chouroulinkov et al., 1988; Steinel and Baker, 1988). The incidences of selected spontaneously occurring neoplastic lesions in CD-I (outbred) and B60171 (hybrid) strains are compared in Table 2.5.

Table 2.4   Normal Body Weights in Grams of Selected Strains of Mice

| Age (Days) | Outbred Strains | | | | | | Inbred Strains | | | | | | Hybrid | |
| | CD-1 | | CF-I | | CFW | | C3H | | C57BU6 | | BALB/c | | B6C3FI | |
	M	F	M	F	M	F	M	F	M	F	M	F	M	F
21	12	11	12	11	9	9	—	—	—	—	—	—	—	—
28	20	18	18	17	16	13	17	16	14	13	16	14	16	14
35	27	22	24	21	19	17	18	17	17	14	17	16	20	17
42	30	24	27	22	24	20	20	18	19	16	18	17	22	18
49	33	26	28	24	27	22	24	23	21	17	20	18	24	19
56	35	27	30	26	28	23	27	26	22	18	21	19	26	21

Source: Data derived from Charles River Laboratories, *Charles River Growth Charts*, 1975.

**Table 2.5   Incidence of Spontaneously Occurring Neoplastic Lesions Lesions Occurring at
Spontaneous Incidence of ~:1% in Either Sex of Charles River CD-1 or 136C3171 Mice Strain**

Location and Lesion	CD-I		B6C3FI	
	M	F	M	F
Lymphoreticular tumors				
Lymphosarcoma			6.0	12.0
Lymphocytic leukemia			1.3	1.4
Lymphoma	3.7	9.9		
Histiocytic lymphoma			0.5	1.4
Histiocytic sarcoma			0.1	1.4
Lymphoblastic lymphoma	1.1	1.7	0.2	0.1
Lymphocytic lymphoma	2.6	1.7	0.6	1.7
Reticulum cell sarcoma	2.6	5.1	0.4	0.2
Skin/subcutis				
Fibrosarcoma	0.2	0.5	1.0	0.5
Mammary gland				
Adenocarcinoma		1.7		0.9
Lung				
Bronchiolar/alveolar adenoma	4.0	2.9	8.3	3.3
Bronchiolar/alveolar carcinoma	3.5	3.1	1.9	0.6
Alveolar type II carcinoma	11.7	13.9	0.2	0.1
Alveolar type II adenoma			2.5	1.2
Adenoma			1.2	0.7
Liver				
Nodular hepatocellular prolif.	5.4	1.7	0.5	0.1
Hepatocellular adenoma	5.6	0.8	17.2	7.1
Hepatocellular carcinoma	7.3	1.0	13.2	2.4
Hemangioma	1.0	1.2	0.7	0.3
Hemangiosarcoma	1.0	0.2	0.5	0.1
Reproductive system				
Ovary				
Cystadenoma		1.1		0.3
Uterus				
Endometrial stromal polyp		3.3		2.9
Endometrial sarcoma		1.9		0.6
Leiomyoma		1.0		
Leiomyosarcoma		1.0		0.3
Hemangioma		1.0		0.9
Pituitary				
Adenoma		3.4	0.3	7.9
Thyroid gland				
Follicular cell adenoma	0.2		0.8	2.4
Adrenal				
Cortical adenoma	8.6	1.0	0.4	0.3
Harderian gland				
Cystadenoma			1.9	1.6
Adenoma			1.5	0.6

Source: Data from Anonymous, *Spontaneous Neoplastic Lesions in the B6C3FIICr]BR Mouse* [control animals
on 24-month studies completed between 1978 and 1986], Charles River Breeding Laboratories,
Wilmington, MA, 1989.

The number of strain-related differences in susceptibility to various test articles and environmental conditions exceeds the scope of this chapter, but additional information is available (Nebert, 1981).

Husbandry

Facilities

Facilities used to conduct toxicology studies in mice must have separate and adequate areas for the required laboratory procedures and for the housing and treatment of study mice. This discussion will be limited to facilities for the housing and treatment of mice. Several characteristics for an adequate facility for the conduct of toxicity studies intended to support regulatory approval of new drugs are listed in the good laboratory practice (GLP) regulations (CFR, 1988a,b). Such a facility should include enough animal rooms or areas to properly separate different species and projects. In addition, there should be facilities for the quarantine of incoming or sick animals and for the isolation of any studies that involve the use of hazardous materials. In practice, animal rooms used for toxicity studies in mice should not contain any other species of animals. Isolation of individual projects is generally interpreted to mean that an animal room should be dedicated to a single toxicity study. One exception to the requirement to conduct only one study per animal room is the case of acute or very short-term toxicity studies, each of which is limited to a small number of animals. Another exception is dermal carcinogenesis, or "skin painting" studies, which generally involve a relatively small number of animals treated for 30–40 weeks. A number of acute or dermal carcinogenesis studies may be run concurrently in a single room. For practical reasons, "adequate isolation" for acute or dermal carcinogenesis studies is typically interpreted to mean separate cage racks for each study and/or isolation of multiple studies within the room by geographical location within the room. The intent of the requirements to isolate species and, in most cases, studies is to reduce the probability of cross-contamination between studies with the various test chemicals or disease entities and to minimize the opportunity for accidental administration of the incorrect test substance to a group of animals. The concept of quarantining incoming animals can be met by having new animals delivered into a sanitized room that contains no other animals, then allowing the new mice an adequate period for acclimatization prior to initiation of the toxicity study. This procedure minimizes the risk of exposure of either new or existing animals to incoming or endemic diseases.

The physical conditions in an animal room are referred to as the macroenvironment. These conditions include such things as the temperature, relative humidity, lighting, ventilation, and concentrations of various gases (e.g., CO_2, ammonia). Many of the macroenvironmental parameters are routinely monitored in facilities that conduct toxicology studies. Abnormal fluctuations in some of these parameters can have a deleterious effect on the validity of toxicity data generated.

Temperature and Relative Humidity

Mice are quite sensitive to variations in temperature and respond to those variations with important physiological changes. This sensitivity is caused by the large-surface-area-to-body-weight ratio in mice, which causes them to radiate heat quickly in a cold environment. Mice respond to low temperature by nonshivering thermogenesis, and resting mice can generate heat at a rate about triple their basal metabolic rate. Group-housed mice can compensate for low temperatures by huddling in a group, a practice that is more effective in a solid-bottom cage containing bedding. Mice have a limited capacity to compensate for excessive heat and do so primarily by vasodilation of the ears to increase heat loss and by increasing body temperature by several degrees. In the wild, mice adapt to excessive temperatures by moving to cool burrows. Mortality is often observed if the ambient temperature reaches 37°C or higher. The range of environmental temperatures where an animal's

oxygen consumption is minimal and virtually independent of changes in ambient temperature is called the thermoneutral zone. The thermoneutral zone for mice is one of the narrowest of species studied and is about $30.0°C \pm 0.5°C$. Mice seem to be generally healthier at a temperature of about 21°C–25°C than they are within the thermoneutral zone (Jacoby and Fox, 1984). The recommended dry-bulb temperature for a mouse housing room is 18°C–26°C (ILAR, 2011).

Variations in environmental temperature can affect the results of toxicity studies in unpredictable ways. Muller and Vernikos-Danellis (1970) found that the acute toxicity of dextroamphetamine was reduced by 10-fold when the temperature was reduced to 15°C from a normal of 22°C, but was increased by two- to threefold when the temperature was increased to 30°C. Conversely, the acute toxicity of caffeine was increased when the temperature was altered either up or down from 22°C. Food consumption is inversely related to ambient temperature, and the secondary effects of changes in food consumption can be complex.

The relative humidity in a mouse room is a significant factor in thermoregulation for the mice housed. At constant temperature, mice are more active at lower relative humidity than at higher humidity. This difference is believed to be a function of the mouse's ability to better dissipate heat under conditions of lower relative humidity (Stille et al., 1968). Low ambient relative humidity has been associated with increased transmission of a disease called ringtail in mice. High relative humidity leads to increased production of ammonia in the urine and feces. Increased ammonia concentrations have been associated with the development of respiratory diseases in rodents (Broderson et al., 1976). The recommended relative humidity for a room housing mice is 40%–70% (ILAR, 1985).

Lighting

Any description of lighting in an animal facility must include a description of the intensity, wavelength or spectrum, and photoperiod of the light.

Light intensity is expressed in footcandles (ftc) (lumen/ft^2) or lux (lumen/M^2). Historically, lighting intensity was selected for the convenience of the researchers, based on the assumption that what was good for people was good for mice. In fact, the *Guide for the Care and Use of Laboratory Animals* in 1978 recommended light intensity of 100–125 ftc. Mice are nocturnal, however, so it is not surprising that their eyes are better adapted to lower light levels. Continuous exposure to light at 100–125 ftc for 6 days has been found to cause loss of 90% of the photoreceptors in albino-beige mice (Robison and Kuwabara, 1978). Light levels of 25 ftc or lower have been recommended for mice (Robison et al., 1982). Amid this discussion of light levels, the specific design and location of individual cages within an animal room can have a substantial effect on the light level at the animal's level. Owing to the nocturnal nature of mice and the attenuation of light by the structure and location of individual cages, there is probably little or no actual injury to an animal housed in a room lighted to 100–150 ftc on a 12-hour light, 12-hour dark cycle.

The wavelength or spectrum of light found in a mouse room is generally a function of the type of fluorescent lighting in common use at the animal facility. While the lighting spectrum may not be of highest concern in designing toxicity studies, Spalding et al. (1969) have shown that mice exhibited the highest level of voluntary wheel-running activity under red light or in darkness, an intermediate level of activity under yellow, and the lowest under green, blue, or daylight. Recalling the nocturnal characteristics of mice, the proximity of red light to dusk/darkness and of blue light to ultraviolet and daylight on the visible spectrum is probably not a coincidence.

The photoperiod for mouse rooms used for toxicity studies is typically diurnal, with a cycle of about 12-hour light to 12-hour darkness. The photoperiod influences circadian rhythms and is probably most often thought of in the context of influence on the estrus cycle. While the estrus cycle of the rat is synchronized by the photoperiod, the estrus cycle of the mouse is less easily influenced by photoperiod (Campbell et al., 1976).

Ventilation

Ventilation in an animal housing facility should be designed to provide sufficient fresh air to remove the thermal load generated by the animals, lights, and equipment; maintain acceptable levels of dust and odor; provide adequate oxygen; and contain any biohazards or intercurrent disease in the animal colony.

A common practice for animal facilities has been to design the heating, ventilation, and air conditioning system to provide 10–15 air changes per hour. In reality, this approach can be quite misleading because it depends upon the total volume of the animal room rather than the biological and thermal load. An animal room with 12 ft ceilings would require 50% more cubic feet of fresh air per minute to achieve 15 air changes per hour than would a room with 8 ft ceilings. Clearly, the more important factor in this example is the number of cubic feet of fresh air per mouse (or kilogram of animals) housed rather than the number of air changes per hour. As a guide, an adequate ventilation rate for a mouse room is about 0.147 ft^3/min of fresh air per mouse, with a heat removal capacity of about 0.6 BTU per hour per mouse (Runkle, 1964).

If at all possible, supplied air should be 100% fresh outside air, introduced into the animal room at ceiling level, with exhaust air picked up at or near the floor to eliminate a maximum amount of heavier-than-air ammonia vapors. Supply air should minimally be drawn through a particle filter, and many facilities now include high-efficiency particle (HEPA) filters on supply air. Addition of HEPA filtration to an existing system will usually require a substantial increase in air conditioning blower power and/or capacity to overcome the added resistance of the filters. Supply of 100% fresh air at the ventilation rates discussed entails significant energy costs for heating and cooling. These costs can be reduced by the installation of a heat (cool) recovery system between the exhaust air and the incoming fresh air. The efficiencies of such a system are accrued during both the heating and cooling seasons. If for some reason, 100% fresh air cannot be supplied, any recycled air must be passed through a complex filtration system to prevent reintroduction of (and cross-contamination by) biological or chemical contaminants and odors.

Relative air pressure between animal rooms and corridors should be considered as a prime mechanism for the control of cross-contamination and communication of disease between animal rooms. In theory, if all animal rooms are either positive pressure relative to the corridor or if all are negative relative to the corridor, there should be no communication of airborne contamination between rooms. If any hazardous substances or human pathogens will be used within the animal rooms, those rooms should be maintained negative to the corridor to prevent contamination of people.

Another subtle consideration concerns the cages or houses of mice undergoing inhalation exposures in closed-bottom cages. Mice will move to the microclimate within the closed-bottom cage that minimizes their exposure to noxious study compounds (Dr. Jerry Finig, D.V.M., personal communication).

Noise

There are no specific regulations or guidelines governing noise to which mice can or should be exposed. There are, however, numerous reports in the literature of adverse or abnormal effects of "stressful" noise on mice, including reduction in body weight (Fink and Iturrian, 1970); changes in immune response and tumor resistance (Jensen and Rasmussen, 1970); audiogenic seizures that are seen in genetically susceptible mice and can also be induced in normal mice; unexpected responses to certain drugs (Iturrian and Johnson, 1975); actual hearing impairment; and others. In view of the variety of effects associated with noise, control of environmental noise in a facility used to conduct toxicity studies in mice should be addressed.

Noise should be described in terms of two dimensions: intensity and frequency. Sound intensity (sound pressure level or loudness) is measured in decibels (dB) and frequency (pitch) is measured

in hertz (Hz). Hertz is the standard unit for cycles per second. While a "safe" intensity of sound has not been described, intensities of 90–100 dB have produced adverse effects, including inner ear damage, and it has been recommended that noise levels be maintained below 85 dB (Anthony, 1963). It is especially important to consider the frequency of environmental noise because the frequency spectrum for hearing in mice differs substantially from that in humans. High-frequency noise that is inaudible to humans can be disruptive or injurious to mice. The average human ear can hear sounds in the frequency range of about 20 Hz–20 kHz, with a maximum sensitivity at about 2 kHz. In contrast, mice cannot hear sounds with frequencies as low as 1 kHz, clearly hear sounds at 50 kHz, and probably have an upper limit in the range of 60–70 kHz (Clough, 1982). Mice emit sound in these upper frequencies, apparently as a means of communication between mothers and young, and associated with mating and aggression. Environmental noise near these frequencies may disrupt normal behavior. Devices that emit sound in this spectrum include ultrasonic motion detectors (used for door openers and intrusion alarms), ultrasonic cleaning and mixing equipment, high-speed homogenizers, dropped or banged metallic devices (e.g., cages, pans, covers), and many others. Dogs and nonhuman primates create noise of an intensity and frequency as to be disruptive to mice.

Environmental noise should be controlled at two levels: at the design and selection of facilities and equipment and at establishment of procedures for animal husbandry and the conduct of laboratory activities that might produce noise at a disruptive intensity or frequency. As the noise of dogs and nonhuman primates is disruptive to rodents (ILAR, 1985), mice should not be housed in close proximity to these species. Loud noises (e.g., barking of dogs) can be transmitted from room to room, sometimes significant distances, through the ventilation system ductwork. This transmission can be reduced by installing labyrinthine configurations and/or commercially available acoustic attenuators in the ductwork that services noisy rooms. Mice should not be housed in close proximity to essentially noisy operations such as cage washing. Intense, high-frequency noise can be generated by the movement of cage racks, equipment carts, etc., in hallways adjacent to animal rooms. A variety of design considerations can improve this situation. A cage rack moving on rubber or synthetic cushioned wheels with well-lubricated bearings over a monolithic flooring will be much quieter than a similar rack on steel wheels with squeaky bearings moving over quarry tile or even concrete flooring. Procedures should be devised, and personnel should be trained with an appreciation for the deleterious effects excessive noise may have on the well-being of mice, and consequently on the results of toxicity studies conducted with those animals.

Construction Parameters

Clearly, a detailed discussion of the architectural and engineering aspects of a facility intended to house laboratory mice is beyond the scope of this text. Our experience has, however, suggested two areas that should receive top priority when new construction, renovation, or even routine maintenance is required.

Cleanability — Animal rooms are exposed to a wide variety of "soil" on a continuing basis. Sources include such things as feed dust, spilled water, animal waste, bedding, parasites (e.g., mouse pinworms), and various bacterial and viral strains that may infect the species housed. Consequently, animal rooms must be swept and mopped frequently, typically using a chemical detergent and disinfectant, and should be sanitized at least before each new study goes into a room, and on a regular basis if a long-term study is in progress. One of the most effective processes for sanitization uses a pressure sprayer to clean ceilings, walls, and floors with an effective disinfectant. This process requires that all surfaces (ceilings, walls, and floors) be coated with a durable, waterproof, chemical-resistant finish. That finish should withstand impacts, such as cage racks colliding with walls, and various objects being dropped on floors without damage to the flooring. In addition, all

lighting fixtures, electrical outlets, switches, computer connections, thermostats, etc., either should be of waterproof construction or should be equipped with waterproof covers that can be closed during sanitization.

Vermin Resistance — Animal rooms are notoriously attractive to insects such as cockroaches, flies, and other pests. The ready access to feed and water that is essential for the mice is an ideal environment for insect infestation as well. The use of toxicants to control pests in rooms housing animals on toxicity studies is discouraged for several reasons. Many common pesticides are known to induce the synthesis of hepatic microsomal enzymes, which in turn may alter the apparent toxicity of the substance being tested. As most toxicity studies are conducted on test substances with unknown or incompletely understood pharmacology, the possible interactions with a particular pesticide are unpredictable. Pests may consume a toxicant, then enter animal cages prior to dying, and be ingested by the study mice. This could lead to indirect intoxication of the study animals.

One environmental requirement for a successful insect population explosion is the availability of a concealed harborage for breeding. We have had remarkable success in controlling insect infestation in our facility by the simple process of sealing, caulking, closing, or eliminating every crack, crevice, hole, wall penetration, electrical outlet, and floor drain in our animal rooms. For this practice to be successful, contractors and maintenance people who work in these rooms must understand the purpose and importance of their task. Cracks in a wall behind a sink are just as useful as harborages as cracks in the middle of the wall, so each contractor and maintenance person must be watching for and eliminating these problems when they are found.

If elimination of harborage has not been implemented or is incomplete, it may be necessary to employ a toxicant on a carefully controlled basis. The study toxicologist should participate in the selection of a suitable toxicant, factoring in all that is known about the pharmacology of the substance being tested in the toxicity study and providing a best estimate of possible interactions with the toxicant. Accurate records should be kept of what toxicant was used, where and how much was applied, and how often it was applied. These factors should be reviewed and considered when the toxicity study is completed and the results are being interpreted.

Caging

The physical conditions in an animal cage (primary enclosure) are referred to as the microenvironment. These conditions (e.g., temperature, relative humidity, lighting, ventilation, and concentrations of various gases [e.g., CO_2, ammonia]) may differ substantially from the conditions in the macroenvironment, depending upon the specific design and placement of the cage within the animal room. Microenvironmental parameters should be evaluated for various cage designs and locations within animal rooms, but are not routinely monitored in facilities that conduct toxicology studies.

Any caging used for mice in toxicology studies should be designed to provide adequate space for freedom of movement and a comfortable environment in terms of temperature, humidity, and ventilation that will minimize stress on the animals. The caging should be cleaned regularly to allow the mice to remain clean and dry. Caging should be as resistant to escape as possible to preserve the integrity of the study as well as the health and safety of the animals. Even if an escaped animal can be recovered and returned to its cage, the health of the animal and the resultant impact on the integrity of the toxicity data generated remain in question, as the animal may have contacted toxic or interfering substances during its travels.

Cage Types

Two types of mouse caging most commonly used for toxicology studies are wire-bottom cages and solid-bottom cages.

Wire-Bottom Cages — Wire-bottom cages are typically suspended in rows on a movable with from 10 to 70 cages arranged on a side. Racks may be single sided or double sided, depending upon the configuration of the animal room and the number of animals that need to be housed. Most contemporary wire-bottom cages are fabricated of stainless sheet steel backs and sides, with stainless steel wire mesh fronts and floors. Sides and backs may have holes or slots stamped into them at manufacture to allow improved ventilation and access to an automatic watering system, if one is available. Mesh fronts allow observation of the animals. The sides of wire-bottom cages are also fabricated of polycarbonate or other rigid plastic product. Transparent plastic cages provide easier observation of animals, but typically offer reduced ventilation through the cage itself. Mesh floors allow urine, feces, and spilled food to drop through, typically to a waste pan or absorbent paper, which can be cleaned or replaced easily and regularly, but have fallen on disfavor due to animals trapping their feet in the mesh. Waste pans should be cleaned or papers replaced at least three times a week. A wire-bottom design has the advantage of keeping animals relatively free of contamination from urine and feces, whereas providing ease of cleaning. Wire-bottom cages should be rotated out of use and washed at least once every 2 weeks. This washing procedure should be monitored regularly and should be adequate to produce negative results on microbiological swab testing. A procedure that achieves good microbiological test results for cages with average levels of soil first requires removal (or emptying) of feeders, waste pans, and water bottles (if present). Then, the racks with the suspended wire cages still in place are passed through a commercial rack washer that operates much like an automatic dishwasher. All cycle times are variable, but an effective combination for average soil is a wash cycle of about 10 min, followed by initial and final rinse cycles of about 3 min each. The wash cycle includes an effective disinfectant detergent (such as PRL-18, manufactured by Pharmacal Research Laboratories, Inc., Naugatuck, Connecticut). All water temperatures (wash, initial, and final rinse) are maintained at or above 82°C (180°F), but this temperature is most important for the final rinse.

Solid-Bottom Cages — Solid-bottom cages, often referred to as shoe-box cages, may be suspended in a rack, like wire-bottom cages, or may be supplied with tops, and arranged on shelves, which are typically movable as a rack. Shoe-box cages are typically constructed of polycarbonate, which allows convenient observation of the animals; polypropylene, which is a translucent plastic; or sheet metal, such as stainless steel. Shoe-box cages provide a secure base for animals and are essential if animals are to be allowed to deliver and suckle live litters. Solid-bottom cages can be provided with filter tops, which can significantly reduce airborne contamination of the environment within the cage. Filter tops do have a negative impact on ventilation within the cage, and levels of CO_2 and ammonia have been found to be substantially higher in cages with filter tops than in those with stainless steel rod tops (Serrano, 1971). There are a number of disadvantages associated with solid-bottom cages. These cages must be provided with some form of low-dust or dust-free absorptive bedding. This bedding must be changed regularly, which is labor intensive. If matings are conducted, vaginal plugs are often difficult or impossible to find in the bedding. As mice engage in coprophagy, a toxic substance or metabolite that is eliminated in the feces may be "recycled," thereby leading to an overestimate of the true toxicity of the substance. In addition, mice commonly ingest various types of bedding, such as wood chips, which renders impossible any serious estimate of food consumption. If an automatic watering system is in use, solid-bottom cages have the potential to fill with water if there is a malfunction, drowning the inhabitant(s).

In routine toxicology studies, solid-bottom cages, covers, feeders, etc., should be rotated out of use and washed once or twice a week, and their supporting racks should be washed at least once per month. This cage cleaning schedule should NOT be followed when reproductive procedures (mating, delivery, and suckling of young) are being conducted, the constancy of home-cage odor is critical to reproductive efficiency, and to reduce the likelihood of cannibalization of young. Cage washing may need to be suspended completely during the period of pregnancy and lactation. (See "Bedding" section of the section for special practices used with reproduction procedures.)

The cage and accessory washing procedure should be monitored regularly and should be adequate to produce negative results on microbiological swab testing. A procedure that achieves good microbiological test results in our facility for cages with average levels of soil employs passage through a tunnel washer on a steel mesh belt. The total transit or cycle time is typically about 3 min, with about 15 s for prewash, and just under 1 min each for main wash rinse, and drying cycles. The wash cycle includes an effective disinfectant detergent (such as Clout, manufactured by Pharmacal Research Laboratories, Inc., Naugatuck, Connecticut). The rinse water is heated to at least 820°C (1800°F), and the drying cycle consists of exposure to high-temperature forced air. The belt speed in a machine such as this can be reduced (total transit time lengthened) for heavily soiled cages or increased (transit time shortened) for lightly soiled cages, but the single criterion that governs the minimum length of the transit time is the maintenance of negative results on the microbiologic monitoring of the clean cages.

Cage Size

Mouse caging must be of adequate size to allow free movement and to avoid overcrowding. Minimum space requirements for mice are provided in the *Guide for the Care and Use of Laboratory Animals: Eighth Edition* (NAP, 2011) on the basis of body weight. Those requirements are in Table 2.6. As a practical matter, to avoid the need for multiple-sized caging and frequent (e.g., perhaps weekly) changes in the size of caging occupied, the minimum specified for adults can be used for all ages.

Population

Mice may be housed singly (one to a cage) or in groups (several animals of the same treatment group caged together) for toxicity testing. It has long been recognized, however, that group housing can substantially alter the toxicity of some substances. Chance (1946) demonstrated that the acute toxicity of a group of sympathomimetic amines was increased by 2- to 10-fold in mice that were group housed compared to mice housed singly. In addition, increased population in solid-bottom cages has been shown to lead to substantially higher levels of CO_2 and ammonia within the cage, even when open stainless steel rod tops were used, but especially if filter tops were in place (Serrano, 1971). Increased levels of ammonia have been associated with hepatic microsomal enzyme induction, which can alter expected metabolism in a toxicity study.

Single Occupancy — Single housing of mice used in toxicity studies offers many advantages. Of paramount importance is the reduced likelihood of mistaking the identity of individuals when conducting various study procedures (weighing, dosing, collecting various observational, or other data). Singly housed mice are more quickly and easily identified and captured throughout the study. In addition, the risk of injury or cannibalism is eliminated in the event that one member of a group becomes debilitated. The biggest disadvantage to single housing is cost. Purchase price

Table 2.6   Minimum Cage Space Requirements for Mice

Body Weight (g)	Floor Area per Mouse		Cage Height	
	in.²	cm²	in.	cm
<10	6.0	38.7	5	12.7
10–15	8.0	51.6	5	12.7
15–25	12.0	77.4	5	12.7
>25	15.0	96.8	5	12.7

for individual caging for large numbers of mice is much higher than the cost for group housing. Individual caging requires much more floor space in the animal rooms than group caging for an equivalent number of animals, and the cost to provide animal care (food, water, cage cleaning, and sanitization) is much higher for single caging.

On the other hand, individually housed animals generally experience greater stress than do group-housed animals. For example, decreases in steroid stress hormones due to social interactions can enhance wound healing (Vegas et al., 2012).

Group Housing — The biggest advantage to group housing is cost savings. Disadvantages include increased probability of mistaken identity, increased stress on animals as a result of establishment and testing of dominance hierarchy, and difficulty with individual animal identification systems. Group-housed mice tend to tear out each others' ear tags, and tattooed markings may be obliterated as a result of repeated fighting for dominance. Measurement of food or water consumption is generally not useful for group-housed mice, as the distribution of food and water among animals of different hierarchical positions tends to be quite uneven. Group housing in solid-bottom cages affects microenvironmental parameters within the cage such as temperature, humidity, various gas concentrations (CO_2, ammonia), and others.

Bedding

Some form of bedding material is required in solid-bottom cages to allow mice to remain clean, dry, and free of urine and feces. The GLP regulations (CFR, 2012) state that bedding should "not interfere with the purpose or conduct of the study" and should "be changed as often as necessary to keep the animals dry and clean." An appropriate bedding should absorb urine effectively, be as free of dust as possible to minimize pulmonary complications, be free of contaminating chemicals, and should have no unacceptable effect on the normal physiology or metabolism of the mice. Inhalation of aromatic hydrocarbons from cedar and pine bedding has been shown to cause induction of hepatic microsomal enzymes, which could seriously compromise the results of a toxicity study (Vesell, 1967; Wade et al., 1968). Opinions differ on whether the use of cedar wood shavings as bedding material contributes to the increased incidence of mammary gland tumors and hepatomas (Heston, 1975; Sabine et al., 1973). One of the most commonly used bedding materials for toxicity studies is hardwood chips derived from woods such as maple, birch, and beech (e.g., Absorb-Dri hardwood chips, Maywood, New Jersey). This selection is probably based on the properties of high absorption and low dust, coupled with an absence of proof that there are harmful interactions.

Bedding material should be changed at least once or twice weekly when cages are washed. More frequent bedding changes may be required if mice are group housed. An exception to this practice for bedding change occurs when reproductive procedures (mating, delivery, and suckling of young) are involved in the study. A continuity of home-cage odor reduces maternal stress, is critical to reproductive efficiency, and reduces the likelihood of cannibalization of young. Therefore, during reproductive procedures, a portion (but not all) of the soiled bedding should be removed at regular intervals and replaced with clean bedding.

Animal Identification

Reliable identification of each animal used in a toxicity study is essential to the integrity of the study data and to the accurate interpretation of study results. The GLP regulations (CFR, 1988d) state that animals, excluding suckling rodents, used in laboratory procedures that require manipulations and observations over an extended period of time or in studies that require the animals to be removed from and returned to their home cages for any reason (e.g., cage cleaning and treatment), shall receive appropriate identification (e.g., tattoo, toe clip, color code, ear tag, and ear punch).

And further that "All information needed to specifically identify each animal within an animal-housing unit shall appear on the outside of that unit." The GLP regulation was amended (Federal Register, 1989) to eliminate toe clipping from the methods listed (earlier) and to discourage the use of toe clipping in nonclinical laboratory studies, as other more humane methods are available. One new method, not listed in the GLP regulation, but which appears to offer some advantages, is the implantable electronic microchip.

Cage Cards

In practice, the comprehensive information specified earlier for the outside of an animal housing unit typically appears on a cage card. This information is relatively straightforward as long as animals are housed singly. The cage card should include complete identification of the study (study number is adequate if a unique coding system is used); identification of or references to the species, strain, sex, source, age, treatment group (e.g., substance tested and dose group); and individual animal number of the mouse (or mice) housed. The identification that appears on the mouse, itself, is limited to a few code letters and/or numbers that can be cross-referenced to the cage card and raw data record for the study to obtain all of the information available about that individual mouse.

Ear Tags

Ear tags are durable, typically made of a noncorroding metal such as Monel, with letters and/or numbers stamped into the surface. The tags are quick and easy to apply, using a special pliers-like applicator, and are easily read over a long period of time. One disadvantage to ear tags is that they can be torn out of the ear if they get caught on something in the cage or, especially, if mice are group housed. Group-housed mice seem to find each others' ear tags especially vulnerable to pulling and hierarchical conflict, and the incidence of tag loss in group-housed mice has proven unacceptably high in our facility. Conversely, the incidence of tag loss in singly housed mice (the norm for toxicity testing at our facility) is remarkably low, especially after the first few days of adaptation. When tags are lost from singly housed mice, they are typically found in or under the cage, so the tag number can be confirmed and a replacement tag installed. A technique to increase tag retention is to avoid the edge of the ear, installing the piercing portion of the tag as near the center of the ear as possible. A useful tag for mice is a small (5/16 in. long) self-piercing Monel tag ("V" shaped prior to application, crimped to a flattened "0" by installation) such as Style 4-1005, Size I from National Band and Tag, Newport, Kentucky. These tags can be ordered custom stamped with at least three numbers or letters on one surface and four on the other surface (i.e., up to 10 million different numbers, more if letters are used as well).

Ear Notching or Punching

Ear notching or punching is a system of holes punched and/or notches cut into the edges of the ears based upon a predetermined numeric code. Figure 2.1 is an example of an ear-marking code (from Leard, 1984). Notching has the advantage of not offering a hard object that can be caught or pulled from the ear, such as an ear tag. Disadvantages include the requirement for substantially more time and care in the initial identification of the animals. Clearly, it takes longer and inflicts more pain on the mouse to punch and clip a precise pattern of one to four or five holes and notches into the two ears of a mouse to crimp on a single ear tag. Imprecisely positioned punches or notches may be hard to read for the duration of the study. Group-housed mice may chew at each others' ears, especially when the notches are fresh, rendering the code more difficult to read. Finally, most codes allow at most a few hundred nonrepeating numbers, meaning that patterns will have to be repeated within most facilities in a short time.
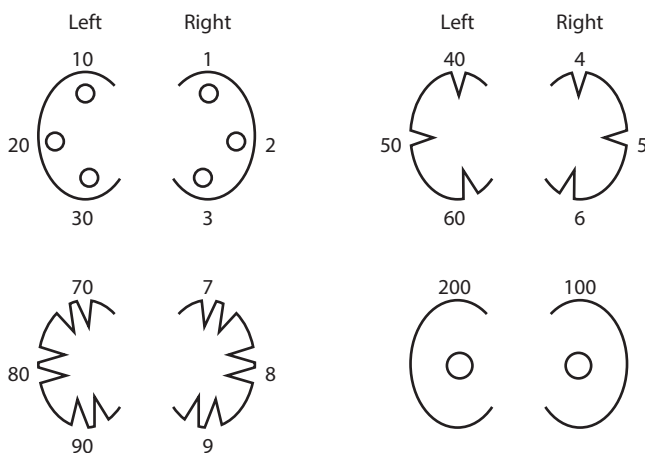
Figure 2.1 Example of an ear marking code which can be used for animal identification.

Tattoos

Mice can be tattooed on the tail or feet as a means of identification. Tattoos are permanent unless they become obliterated by chewing or injury. The process of applying alphanumeric tattoos to the tails of mice is more time consuming than ear tagging or notching, but more accessible than the feet. Another practical consideration is the size of a mouse tail. Very small letters are both more difficult and time consuming to apply to a mouse tail, and more difficult and time consuming to read at each subsequent identity check throughout the study. The size of the mouse tail and practical considerations of legibility limit the tattoo to four to five alphanumeric characters on the dorsal (easily readable) surface of the tail. It is possible to tattoo an additional four to five alphanumeric characters on the ventral surface of the tail if necessary. This surface is less easily read.

The most common long-term approach to animal identification is the implantable electronic microchip that can be used for study or facility code numbers that need not be read at each identity check.

While tattooing is a viable method of identification for longer toxicity studies because of its permanence, the amount of time required to both apply and read the tattoos makes this method impractical for shorter studies.

Color Coding of Skin or Hair

The skin or fur of a mouse can be quickly color coded using a variety of indelible felt-tip markers. A variety of combinations of colored markings on the tail or on the fur of the back are easily read without disturbing the animal. This procedure is particularly useful for toxicity studies of short duration. Owing to the fastidious grooming habits of mice, there is a tendency for colored marks to be groomed off quite quickly. This problem is exacerbated if the mice are group housed. A number of different brands and types of markers should be tested, as there is wide variability in their "durability" or resistance to grooming. This problem can be reduced somewhat by locating the color code in a place that is more difficult to groom, such as the top or back of the head. One substance that confers a durable stain on the fur is Bouin's fixative, but the color is limited to yellow, so the identity code must be based upon the location of the stain rather than the color. The number of possible easily distinguished colors and combinations of colors available for a color code and the maximum number of different sites that can be easily coded on a mouse limit the number of unique codes to a few hundred.

In general, color coding is a useful procedure for identifying mice in short studies. The toxicologist should evaluate each mouse and be prepared to "touch up" the color code on a daily basis.

Toe Clipping

Toe clipping for purposes of identifying mice involves amputation of various combinations of toes using a surgical scissor, nail clipper, or other suitable device to establish an identity code. Toe clipping has the advantage of permanence, but an individual may become "unreadable" through accident or injury that compromises unclipped toes. Toe clipping has many disadvantages and no clear advantages over other methods such as ear tagging or notching. Most importantly, toe clipping is more traumatic than other methods and has an increased likelihood of resulting in infection because it involves the feet. The method is time consuming to perform, and the codes are difficult to read through the duration of the study because the mouse must be picked up, and often the toes of each foot must be spread to facilitate accurate reading. As stipulated at the beginning of this section on animal identification, the FDA has amended the GLP regulations to eliminate toe clipping from the list of procedures recommended for identifying animals. Toe clipping may have some utility in neonatal (or very young) mice, in which the nervous system is not as well developed, and more conventional methods are impractical.

Implantable Electronic Microchips

The current preferred approach to animal identification is the implantable electronic microchip. The microchip is a transponder that has been sealed into a glass capsule about 1.0 cm in length and about 1.5 mm in diameter. This device is suitable for subcutaneous (sc) implantation in mice. The device is "energized" by one of a variety of portable or stationary readers, which emits a low-power radiofrequency signal. The transponder is stimulated to transmit its unique identification number back to the reader, where it can be both displayed and linked directly to a computer system. The microchips are easy to install, resistant to loss, and should perform well over the course of a longer-term study. The device has a capacity for up to 34 billion different numbers. Preliminary data from several laboratories suggest that the devices are well tolerated and do not produce problematic histological changes at the implantation site. One disadvantage is that the devices must be read by electronic reading equipment. The implication is that in the event of equipment failure, there is no means for manual decoding or reading of the chips. Possession of multiple readers should provide reasonable redundancy for most facilities.

Food

Nutritional Requirements

The explicit nutrient requirements for mice have been neither extensively studied nor defined. What is known of these requirements has been estimated on the basis of a number of studies that have had other objectives. Some studies have focused on the effects of specific dietary deficiencies, and others have looked at "acceptable performance" in growth and reproductive parameters as evidence of nutrient adequacy. Mice have different nutrient requirements for growth, reproduction, and maintenance, and the many diverse genetic strains differ in their minimal requirements as well. Estimated minimum nutritional requirements for proper growth and reproduction of "conventional mice" have been compiled in Table 2.7 (NRC, 1978). The estimated nutritional requirements for laboratory mice are published periodically by the National Research Council in a document titled *Nutrient Requirements of Laboratory Animals* (National Academies Press, 1995). This document

Table 2.7   Estimated Nutrient Requirements of Mice

Nutrient	Unit	Requirement
Linoleic acid	%	0.3
Protein (growth)	%	12.5
Protein (reproduction)	%	18.0
L-Amino acids		
Arginine	%	0.3
Histidine	%	0.2
Isoleucine	%	0.4
Leucine	%	0.7
Lysine	%	0.4
Methionine	%	0.5
Phenylalanine	%	0.4
Threonine	%	0.4
Tryptophan	%	0.1
Valine	%	0.5
Minerals		
Calcium	%	0.4
Chloride	(required, but not quantified)	
Magnesium	%	0.05
Phosphorus	%	0.4
Potassium	%	0.2
Sodium	(required, but not quantified)	
Chromium	mg/kg	2.0
Copper	mg/kg	4.5
Fluoride	(status uncertain for mice)	
Iodine	mg/kg	0.25
Iron	mg/kg	25.0
Manganese	mg/kg	45.0
Selenium	(required, but not quantified)	
Vanadium	(status uncertain for mice)	
Zinc	mg/kg	30.0
Vitamins		
A	IU/kg	500.0
D	IU/kg	150.0
E	IU/kg	20.0
K_1 equivalent	mg/kg	3.0
Biotin	mg/kg	0.2
Choline	mg/kg	600.0
Folacin	mg/kg	0.5
Inositol (myo-)	(bacterial synth. usually adequate)	
Niacin	mg/kg	10.0
Pantothenate (Ca)	mg/kg	10.0
Riboflavin	mg/kg	7.0
Thiamine	mg/kg	5.0
Vitamin B_6	mg/kg	1.0
Vitamin B_{12}	mg/kg	0.01

Source: National Research Council (NRC), *Nutrient Requirements of Laboratory Animals*, 10, 3rd revised ed., National Academy of Sciences, Washington, DC, 1978.

considers most topics related to mouse nutrition. This document is revised when important new information becomes available and is considered to be a current, definitive source.

Selection

The choice of a specific diet to be used in a mouse toxicity study should take into account all of the objectives and requirements of the study as well as the convenience, efficiency cost, and availability of a particular diet. In general, diets obtained from well-known commercial suppliers with established procedures for quality control and documentation will be worth any added cost. One of the basic choices is whether to use pelleted diet or meal. Pelleted diet is generally easier and neater to handle, both for the people and for the mice involved. The meal form of a diet is usually easier to use if the study design requires mixing of a test chemical with the diet, or if food consumption will be measured.

Mice can obtain adequate nutrition from a variety of types of diets. Diets are classified on the basis of the amount of refinement of the ingredients (NRC, 1978). Three types of diets will be described here. The types are natural ingredient diets, purified diets, and chemically defined diets.

Natural Ingredient Diets — These types of diets contain grains such as corn, wheat, oats, beet pulp, and other ingredients that have been subjected to minimal processing such as fish meal, soybean meal, wheat bran, and a variety of vitamin and mineral supplements. They also have been referred to as a cereal-based, unrefined, nonpurified, or stock diets. Natural ingredient diets are the most widely used and are relatively economical. The principal objection to such diets is that they have had a tendency to vary widely in terms of nutrient and contaminant content (Newberne, 1975; Rao and Knapka, 1987; Wise, 1982; Wise and Gilburt, 1980). Data have been compiled on key nutrient and contaminant concentrations for all of the lots of a commercially available natural ingredient rodent diet (Purina Certified Rodent Chow 5002, Purina. Mills, Inc., St. Louis, Missouri) received at this facility over a period of 1 year. The variability of these concentrations from lot to lot is relatively low, as seen in Tables 2.8 and 2.9.

Purified Diets — These types of diets are formulated exclusively with refined ingredients. Dietary protein may be derived from sources such as casein or isolated soy protein, carbohydrate may be derived from sugar or starch, fat may be derived from vegetable oil or animal fat, and dietary fiber may be derived from some form of cellulose. Inorganic salts and pure vitamins are added to provide essential vitamins and minerals. Purified diets also have been referred to as sernipurified, synthetic, or semisynthetic. They offer consistent nutrient concentrations and the ability to modify those concentrations to achieve specific nutritional deficiencies or excesses. Purified diets are less palatable for animals, and food consumption should be monitored prior to study initiation and during the study to assure that adequate nutrition is being maintained.

Chemically Defined Diets — These types of diets are formulated entirely with chemically pure compounds. Amino acids, sugars, triglycerides, essential fatty acids, inorganic salts, and vitamins are blended to provide appropriate nutrition. These diets offer strict control of specific nutrient concentrations at the time of manufacturer, but the bioavailability of those nutrients may be altered by oxidation or interaction among nutrients. The availability of specific nutrients to the mice, then, may not be what the toxicologist believes is being providing. Chemically defined diets have the further disadvantages of being difficult to formulate and have a very narrow appeal in toxicity testing.

In addition to the degree of refinement of ingredients, mouse diets are classified as closed formula or open formula on the basis of the availability of the quantitative as well as the qualitative aspects of the feed blend.

**Table 2.8   Content of Several Key Nutrients in Different Lots of a Closed-Formula,
Natural Ingredient Rodent Diet (Purina Certified Rodent Chow 5002)**

	Protein (%)	Fat (%)	Fiber (%)	Calcium (%)	Phosphorus
Pellets	20.8	5.75	4.46	0.781	0.680
	20.3	4.64	4.20	0.708	0.650
	20.3	5.08	4.14	0.684	0.633
	21.6	5.45	4.76	0.834	0.666
	20.3	5.40	4.28	0.893	0.661
	21.9	4.93	4.11	0.758	0.695
	20.8	5.00	4.60	0.774	0.588
	20.2	5.73	4.09	0.710	0.629
	20.1	5.82	3.77	0.760	0.607
	21.0	6.07	3.97	0.786	0.683
	20.2	5.94	4.36	0.768	0.599
	20.8	5.89	4.39	0.831	0.745
	20.6	5.80	3.95	0.841	0.592
	20.5	5.60	4.08	0.682	0.592
	20.3	5.74	3.75	0.763	0.606
	20.7	5.68	3.86	0.853	0.622
	20.3	5.90	3.87	0.726	0.536
	20.8	5.50	3.74	0.754	0.588
	20.8	6.17	3.81	0.860	0.600
Means	*20.65*	*5.58*	*4.12*	*0.777*	*0.630*
Meal	21.3	5.76	4.47	0.850	0.589
	21.9	5.45	4.45	0.924	0.716
	21.5	5.40	4.26	0.715	0.542
	21.7	4.82	3.84	0.877	0.696
	21.4	4.92	4.07	0.718	0.647
	0.3	5.74	4.00	0.632	0.561
	20.3	5.91	4.31	0.903	0.566
	20.7	6.43	3.68	0.741	0.676
	21.1	5.83	4.13	0.809	0.701
	21.1	6.02	4.03	0.681	0.587
	21.2	5.76	4.45	0.817	0.732
	21.2	6.15	3.99	0.880	0.576
	21.1	5.75	3.67	0.849	0.581
	21.5	6.33	3.93	0.832	0.605
	20.5	6.16	4.20	0.732	0.561
	20.4	5.81	3.97	0.790	0.602
Means	*21.08*	*5.77*	*4.09*	*0.797*	*0.621*
Combined means	*20.84*	*5.67*	*4.10*	*0.786*	*0.626*
Range	*20.1–21.9*	*4.64–6.43*	*3.67–4.76*	*0.632–0.924*	*0.536–0.745*

Table 2.9 Content of Several Key Contaminants in Different Lots of a Closed-Formula, Natural Ingredient, Rodent Diet (Purina Certified Rodent Chow 5002) (in ppm)

	Arsenic	Cadmium	Lead	Mercury	Selenium
Pellets	0.334	0.0632	0.247	<0.05	0.119
	<0.200	0.0499	0.188	<0.05	0.190
	<0.200	0.0362	0.142	<0.05	0.221
	0.206	0.0711	0.139	<0.05	0.214
	<0.200	0.0647	0.136	<0.05	0.231
	<0.200	0.0620	0.138	<0.05	0.171
	<0.200	0.0779	0.181	<0.05	0.267
	<0.200	0.0551	0.132	<0.05	0.199
	<0.200	0.0838	0.146	<0.05	0.223
	0.210	0.0761	0.273	<0.05	0.267
	<0.200	0.0747	0.136	<0.05	0.243
	<0.200	0.0755	0.132	<0.05	0.176
	<0.200	0.0907	0.156	<0.05	0.243
	0.269	0.107	0.246	<0.05	0.178
	0.263	0.107	0.266	<0.05	0.214
	<0.200	0.111	0.147	<0.05	0.250
	<0.200	0.109	0.144	<0.05	0.161
	0.224	0.104	0.202	<0.05	0.205
	<0.200	0.105	0.147	<0.05	0.260
Means		*0.0821*	***0.174***	*<0.05*	*0.212*
Meal	0.255	0.0514	0.348	<0.05	0.258
	<0.200	0.0677	0.233	<0.05	0.280
	<0.200	0.0919	0.155	<0.05	0.283
	0.403	0.0498	0.145	<0.05	0.236
	<0.200	0.0427	0.214	<0.05	0.156
	<0.200	0.0520	0.147	<0.05	0.217
	<0.200	0.0804	0.117	<0.05	0.253
	0.321	0.0700	0.296	<0.05	0.208
	<0.200	0.0677	0.150	<0.05	0.212
	<0.200	0.0831	0.105	<0.05	0.224
	<0.200	0.0718	0.150	<0.05	0.228
	<0.200	0.0824	0.164	<0.05	0.216
	<0.200	0.122	0.131	<0.05	0.197
	<0.200	0.109	0.268	<0.05	0.214
	<0.200	0.120	0.153	<0.05	0.193
	<0.200	0.120	0.146	<0.05	0.199
Means		*0.0801*	*0.183*	*<0.05*	*0.223*
Combined means		*0.0802*	*0.178*	*<0.05*	*0.217*
Range	*<0.200–0.403*	*0.0362–0.122*	*0.105–0.348*	*N/A*	*0.119–0.283*

Closed-Formula Diets — Manufacturers and vendors of closed-formula diets typically provide a list of ingredients used to manufacture the diet, but not the actual proportions of each ingredient in the final blend. They also typically provide target or mean nutrient analyses from a number of batches. A reputable commercial vendor will analyze feed in production and make minor adjustments to the quantitative aspects of the blend on a frequent basis to maintain nearly constant concentrations of key nutrients such as protein, fat, fiber, and minimal concentrations of potentially important contaminants (Ettle, n.d.).

Open-Formula Diets — These types of diets are typically based on a published formulation for the quantity of each nutrient to be included, for example, ground wheat: 230 g/kg, ground corn: 245 g/kg, and soybean meal: 120 g/kg. An example of an open-formula, natural ingredient diet (Knapka et al., 1974) is in Table 2.10. It is often assumed that open-formula diets provide more consistent nutrient and contaminant concentrations than equivalent closed-formula diets because the relative proportions of ingredients stay fixed among lots and across time. The opposite may be true. While it is true that the diet described in Table 2.10 will always contain 230 g/kg of ground wheat, it is also true that the actual protein and contaminant contents of that wheat (and of many other constituents) may vary widely during the year, depending upon climatic conditions during growth, geographical location of the source, and storage conditions since harvest (Greenman et al., 1980). These variables also affect lots from year to year.

Key nutrient and contaminant concentrations of an open-formula natural ingredient diet (3–94 different lots of NIH-07) (Rao and Knapka, 1987) are compared to those of a commercially available closed-formula diet (35 lots of Purina Certified Rodent Chow 5002) in Table 2.9. Based upon the relative ranges of values for each of the constituents compared in this example, the closed-formula diet was substantially less variable than the open-formula diet in every constituent compared.

Provision of Feed and Feeders' Feed

Feed is typically provided on an *ad libitum* basis for toxicity studies. Healthy adult mice will consume about 4–5 g of feed per day, and ordinarily they should be given a quantity adequate for at least 3–4 days. Typically, quantities sufficient to last more than a week are provided, with the balance discarded and replaced about weekly to maintain freshness. Two notable exceptions to *ad libitum* feeding are immediately prior to oral dosing in acute toxicity studies and prior to blood collection for clinical laboratory analysis in studies of any duration.

The process of fasting mice presents a dilemma. Mice are nocturnal, and therefore, when food is available *ad libitum*, most of what they consume during a 24-hour period will be consumed during the dark phase of the light/dark cycle. Their stomachs are normally quite full in the morning and empty slowly through the day. Any fasting procedure that begins in the morning and continues for up to about 12 hours will result in a nutritional state (based on average stomach contents) that does not differ appreciably from fed mice. The next longer convenient interval is to fast animals overnight, from the end of one working day until the morning of the next (a period of about 15–16 hours, which includes the normal feeding time). Overnight fasts are probably the norm for toxicity studies in mice. If food is available, young adult, outbred albino mice (25–30 g) will gain about 7%–10% of their "afternoon" body weight owing to feed consumption during that 16-hour period. If food has been removed, those same mice will lose about 8%–10% of their "afternoon" body weight as a result of the fast (data from our laboratory). Fasting mice for a period of 24 hours results in about a 30% reduction in absolute liver weight (liver/body weight ratios stay constant), a reduction of over 90% in hepatic glycogen content, and about a 50% reduction in reduced hepatic glutathione, but an increase of about 150% in total hepatic triglycerides (Strubelt et al., 1981). These changes were associated with increased hepatotoxicity from a variety of xenobiotics, and at least one author (Strubelt) believes that overnight fasting of mice should be avoided. The dilemma, then, is that if mice must be fasted,

Table 2.10   Open-Formula Natural Ingredient Mouse Diet

Ingredient	Amount per Ton
Major ingredients (lb)	
Dried skim milk	100
Fish meal (60% protein)	200
Soybean meal (49% protein)	240
Dehydrated alfalfa meal (17% protein)	80
Corn gluten meal (60% protein)	60
Ground #2 yellow shelled corn	490
Ground hard winter wheat	460
Wheat middlings	200
Brewer's dried yeast	40
Dry molasses	30
Soybean oil	50
Dicalcium phosphate	25
Salt	10
Ground limestone	10
Mineral and vitamin premixes	5
Mineral premix (g)	
Cobalt (cobalt carbonate)	0.4
Copper (copper sulfate)	4.0
Iron (iron sulfate)	120.0
Manganese (manganese oxide)	60.0
Zinc (zinc oxide)	16.0
Iodine (potassium iodate)	1.4
Vitamin premix	
Vitamin A (IU)	5,500,000
Vitamin D3 UU)	4,600,000
a-Tocopheryl acetate (TU)	20,000
Vitamin K (menadione sodium bisulfite) (g)	2.8
Choline (choline chloride) (g)	560.0
Folic acid (g)	2.2
Niacin (g)	30.0
D-Pantothenic acid (calcium pantothenate) (g)	18.0
Riboflavin (g)	3.4
Thiamine (g)	10.0
Vitamin B_{12} (Ag)	4000.0
Pyridoxine (g)	1.7

Source: Knapka, J. J. et al., *Lab. Anim. Sci.*, 24, 480, 1974.

whether they should be fasted for a few hours during the day, which will be unlikely to achieve the needed effect, or overnight, which may have considerable effect on hepatic parameters.

Feeders — Feed is provided to mice in one of a very wide variety of feeders. One manufacturer alone (Lab Products, Inc.) lists over 25 different feeders suitable for mice. The mere existence of such a variety of designs testifies to the fact that no single design exists that satisfactorily solves all of the problems encountered in providing clean, dry feed to mice. Some of the basic problems include the fact that mice, if given the opportunity, will urinate, defecate, and sleep in their feeders as well as dig in it, play with it, and in almost any other conceivable way distribute feed outside of their feeders and even their cages. The challenge in feeder design, then, is to keep the mice out

of (and off the top of) the feeder, minimize spillage (especially of meal), and still provide free access to the feed at all times. This challenge is more easily met for pelleted diet, especially when feed consumption is not being measured. In that situation, a feeder that is enclosed at the top and provides access to the feed through some form of slots of wire mesh at the bottom and sides is effective.

The more common situation in toxicity testing is that feed consumption will be measured. While this can be done with pelleted feed, and an inexpensive feeder has been reported (Dunn and Stem, 1978), meal is the more common dietary form when feed consumption will be measured. Feeders containing meal must be easy to install and remove from cages without spillage to facilitate weighing. They must be heavy enough and fit securely enough into the cage to prevent tipping or movement. A common form consists of a small glass jar or stainless steel bowl with a restrictive top plate to limit ease of entry. Various forms of feed followers (heavy washers with several holes, wire mesh, even marbles) can be placed on the surface of the feed to discourage digging or other means of expulsion of the feed.

Analysis

If toxicity studies are being conducted to support a safety claim to a regulatory agency (such as the USFDA), the GLP regulations should be considered. The GLP document (CFR, 1988c) stipulates that feed be analyzed periodically for levels of contaminants that might interfere with the toxicity study and that documentation of such analyses be available. This consideration is particularly important for natural ingredient diets, which will almost certainly contain at least trace amounts of a variety of heavy metals, pesticides, and other environmental pollutants. The chemistry of the test article being studied should be evaluated for any special sensitivities to common contaminants, and if those sensitivities appear likely, special care should be taken to avoid those contaminants. In the absence of any suspected special sensitivities, diet should be screened on a regular basis for the well-known environmental contaminants such as heavy metals, aflatoxins, and various pesticides. One of the most practical (and common) solutions to the requirement for routine feed analysis is to purchase what is referred to as "certified" feed from one of the major commercial suppliers that offer such products. While more expensive than ordinary feed, certified feeds are analyzed for common contaminants by the supplier, and each lot is typically supplied with documentation of the results of those analyses that should meet regulatory requirements.

Water

Mice should be provided with *ad libitum* access to a source of clean fresh water. For most toxicity studies, ordinary potable water available from a typical municipal water supply is appropriate. Some animal facilities are equipped to treat drinking water to alter pH or to reduce chemical or microbiological contaminant levels. Treatment may include chlorination, reverse osmosis, distillation, or ion exchange.

Little is known about the mean quantitative water requirements for mice, but quantities of 6–7 mL/day are thought to be representative for adults (Jacoby and Fox, 1984). It is known that environmental temperature is the primary factor influencing water requirements (Knapka, 1983), with higher temperature and/or lower humidity leading to increased water consumption. The type of diet may also affect water consumption. The mouse has a biological half-time for turnover of water of 1.1 days, which is shorter than that in larger animals (Jacoby and Fox, 1984). Owing to this short half-time, mice are particularly sensitive to water deprivation and, if they are maintained on a dry diet, may die within as little as 1 day of water deprivation. Any restriction in the availability of water results in an immediate and dramatic reduction in food consumption and commensurate

fecal output. This relationship is so reliable that a toxicology technician trained to be aware of reductions in fecal output on a regular basis will almost never miss a malfunction or deficiency in a watering system or feed source.

Water is typically distributed to individual caging by one of two methods: water bottles with sipper tubes attached to each cage or an automatic watering system.

Water Bottles with Sipper Tubes

This type of watering device has the advantages of lower initial purchase cost and the ability to estimate water consumption on a per-cage basis. Bottles have several disadvantages, including higher maintenance or use cost (bottles must be removed, disassembled, washed, refilled, and reinstalled at least once or twice per week to minimize bacterial contamination), some configurations make access to suspended caging more difficult, stoppers must be carefully installed to prevent leakage with resultant water deprivation, and some configurations require very careful installation of the bottle on the cage to assure that sipper tubes have an adequate downward slope to preclude air-lock and resultant water deprivation. Some cage designs allow mice to chew on water bottle stoppers, with the potential for ingestion of the debris.

Automatic Watering Systems

Such systems carry high initial purchase (and installation) costs and generally lack the capacity to indicate water consumption per cage. Automatic watering systems for both the room and the cage rack distribution systems are usually constructed of stainless steel or PVC piping with stainless steel fittings. Stainless steel is more expensive, but also more durable. Automatic systems are supplied by some type of pressure reduction station connected to the facility water supply, such that the water pressure at the cage is in the range of 1.5–4.0 lb/in.[2] (psi). The pressure-reduction station can be fitted for automatic system monitoring (e.g., for leaks, overly high or low distribution pressure) and can provide connections for local water treatment such as chlorination. Racks are connected to the room distribution system by means of flexible hosing and may include an air-gap isolator to prevent microbial contamination front traveling from a rack into the distribution system. Automatic watering systems require less labor while in use than water bottles, being limited to periodic checks that individual sipper fittings are functioning properly and that air has not been trapped in the rack distribution system. Whenever a rack is connected to the system, whether new to a room or reconnected after relocation, the rack should have all air bled from its distribution.

System and representative sippers should be checked to confirm the absence of air in the lines. Room distribution systems, particularly those in rooms with a small number of resident animals, have the potential to foster bacterial growth in the distribution system. This problem seems least severe in those rooms with the highest daily water flow rates. Automatic watering systems can be fitted with a central flushing system that provides a programmed flushing of the room distribution system at increased system pressure and flow rate. A program of daily flushing for about 5 min has been found to produce an acceptable level of microbiological control.

Water Quality

This should be monitored by a regular system of chemical and microbiological analyses. Samples should be collected from water bottles that have been in use for nearly the maximum allowable period, from clean bottles, and from the water source. Samples from automatic watering systems should be collected at sippers that have been in use in individual cages, at clean sippers, at one or more sources on the animal room distribution system, and at both the high- and low-pressure sides of the pressure-reduction station. Water samples should be analyzed for the routine

contaminants (e.g., heavy metals, pesticides) and for microbiological content (both type and plate count or concentration). Any special requirements of the toxicity study should be considered when selecting parameters for analysis. In general, water that is of acceptable quality for human consumption will be acceptable for mice.

Prevention of Infectious Diseases

The occurrence of an infectious disease during the conduct of a toxicity study will at best confer uncertainty about the interpretation of the study results and at worst require the repetition of the entire study. The effect of even relatively benign infections on the health of mice that have been compromised by the administration of high doses of a potentially toxic test article can rarely be accurately predicted.

This section will discuss some of the fundamental considerations in the prevention of infectious disease in a mouse colony. A comprehensive discussion of infectious, neoplastic, and noninfectious diseases in mice is beyond the scope of this chapter, but has been addressed elsewhere (Foster et al., 1982; Jacoby and Fox, 1984).

Simplistically, infectious diseases can be prevented by obtaining high-quality healthy mice from a reputable supplier, protecting those mice from exposure to infectious disease, and maintaining those animals in a clean, well-controlled environment with good quality food and water.

It is fair to assume that the health status of mice will be best at the time they leave the breeding colony. Some commercial breeders are now offering mice that are free of antibodies to specific lists of mouse viruses as well as most important bacteria and parasites. These animals may be especially susceptible to infectious disease precisely because they lack antibodies to most common mouse diseases.

Periods when healthy mice are especially subject to exposure to infectious disease include the transit from the breeder to the user and the introduction into the new home colony. Risk during transit is increased if the animals are transported by common carriers, such as commercial airlines and trucking companies. Often, common carriers will be less cautious with animals than trained staff. These risks can be reduced if mice are shipped in filtered containers that exclude much of the biological contamination to which the container is exposed and if they are shipped in a clean, environmentally controlled vehicle without intermediate transfer between shipper and receiver.

The largest risk upon receipt of mice at the new home colony is that the mice will be transported through or into an area that is inhabited by animals that are already infected with a contagious disease. This risk can be reduced by maintaining the integrity of the filtered shipping carton until the mice reach the room in which they are to be housed. The room should have been previously sanitized to reduce the probability of infection from previous residents. It is generally a poor practice to introduce new mice into an animal room that already contains animals, especially if those animals have been in-housed for any period of time or were received from another supplier. Different species should never be mixed in the same room. Environmental conditions, including temperature, humidity, and ventilation, and their contribution to animal health have been discussed earlier in this chapter.

Once clean healthy animals have been introduced into a clean room stocked with clean caging, food, and water, their health status becomes a function of the quality and training of the animal care staff. A good understanding of the biology of mice, of infectious disease, and of the kinds of things that can act as vectors for potential infection is an invaluable asset for animal care staff. Handlers should be encouraged to wear gloves and laboratory coats or uniforms when working with mice and to change those garments regularly. Proper hygiene is especially important when moving from one animal to another. In the event that an infectious disease is diagnosed in the colony, every reasonable effort should be made to isolate (or even eliminate) the infected animals to prevent spread. This might include maintenance by a handler or handlers who do not come into contact with important study animals.

Study Design

Most toxicity and teratology studies conducted in mice are designed to provide information on potential human toxicity. Test substances are typically administered by the expected route of human exposure. A pharmaceutical product that is intended for oral administration (tablet, capsule, solution, or suspension) or a food additive would generally be administered by the oral route. Oral administration to mice is usually accomplished by administration of a solution or suspension by oral gavage, by mixture of the test substance with the diet, or less commonly added to the drinking water.

The specific design of toxicity studies should be tailored to the objective to be achieved and to any specific characteristics of the test substance. Many features of study design will be predicated on guidelines and practices of regulatory agencies such as the FDA or Environmental Protection Agency (EPA) in the United States or their counterparts in other countries to which the results of the "4902MIN" submitted in support of a safety claim. Recommendations for study length (duration of dosing) fall in this category.

Toxicity studies are usually conducted in the order of increasing duration of dosing, beginning with acute toxicity studies. When this regimen is followed, each study provides progressively more useful information for the selection of doses for the next longer study.

Acute Toxicity Studies

Acute toxicity studies are conducted to evaluate the effects of a single substance. Usually, each animal receives a single dose of the test substance in this study design. On rare occasion, repeated doses may be administered, but in any event, all doses are administered within 24 hours or less. Historically, a primary objective of acute toxicity testing was to determine an LD50 dose or that dose that would be lethal to 50% of the animals treated. To achieve this objective, groups of mice, often numbering 10 or more per sex, are treated with a single dose of the test substance. Depending upon the rate of survival in the initial group(s), additional groups are added to the study at higher and/or lower doses such that most animals die that receive the highest doses and most survive that receive the lowest doses. Survival is assessed at some predetermined interval after dosing, usually 7 or 14 days, but occasionally as early as 24 hours. The resultant dose–response data can be analyzed by a statistical method such as probit analysis (Finney, 1971) to provide an estimate of the median lethal dose (LD50) and some measure of the precision of that estimate, such as the 95% fiducial limits. There are very few scientifically valid reasons to include determination of the LD50 as a significant objective of acute toxicity testing. Most regulatory agencies have dropped their requirements for a specific value for the LD50, and animal welfare considerations preclude the use of the large numbers of animals previously required.

A more contemporary design for acute toxicity testing attempts to derive a maximum amount of information from a minimum number of animals. Study objectives include determination of the most important clinical signs attributable to high doses of the test substance, time of onset and remission of those signs, possible determination of a minimum lethal dosage, and in the event of lethality, the sequence and timing of effects leading to death or recovery. These objectives are achieved by means of a comprehensive schedule of animal observations following dosing. These objectives can usually be achieved by treating from one to three groups of three to five mice/sex/ group at different doses.

Traditionally, acute toxicity testing of potential new pharmaceutical products is conducted in at least three species, with one being a nonrodent, and by at least two routes of administration, one of which is the intended clinical route. Mice are the most frequently selected rodent species for acute toxicity testing. The choice of routes of administration depends on the intended clinical

route and on how much is already known about the oral bioavailability of the test substance. If the intended clinical route is oral, acute testing by oral gavage with a solution or suspension is of primary importance. If other clinical routes are anticipated (e.g., intravenous [iv] or dermal), they represent good secondary routes for acute testing. Ordinarily, at least one parenteral route is used for acute testing and that route may be *IV* if the product is soluble in a fairly innocuous vehicle (e.g., water or saline) or intraperitoneal (ip) as a suspension if the product is insoluble in an aqueous (or other innocuous) vehicle. If the intended clinical route is not oral, the oral route is usually selected as a secondary route for acute toxicity testing to provide information relevant to accidental oral ingestion. A rough estimate of oral bioavailability can be based on a comparison of the acute toxicity associated with various doses administered by the oral and parenteral routes. Acute toxicity testing conducted for other purposes is usually more limited in scope. Most regulatory agencies no longer require a full complement of species and routes of administration to render decisions on acute toxicity.

There are a few characteristics of acute toxicity testing that are not common in other toxicity protocols. In a typical repeated-dose toxicity study, several groups of animals are treated concurrently with predetermined doses of test substance and a control substance. To reduce animal use in acute toxicity testing, studies that include more than one dose group are usually dosed sequentially, with an interval of at least 24 hours between dosing of subsequent groups. This allows the effects of the previous dose to be fully manifested and allows selection of the subsequent dose to provide the highest probability of contributing more useful information. Another unusual aspect of acute toxicity studies is the nutritional status of the animals at dosing. Because some schools believe that the results of acute toxicity testing are more reliable if all animals are in a uniform nutritional state, mice to be dosed orally are often fasted overnight prior to dosing. Fasting allows dose volumes to be higher than in repeated-dose studies, and because dosing occurs only on the first day, dietary stress is considered tolerable. The scientific merits of this practice are debatable, but fasting is "traditional" in oral acute toxicity studies. While the practice of conducting gross necropsies at the end of acute toxicity studies is growing in popularity, this practice rarely yields useful information. The toxicity resulting from acute exposure is usually associated with a biochemical or functional imbalance rather than with a change in the gross or microscopic architecture of an organ system. Changes observable at gross necropsy are more often associated with repeated dosing at sublethal levels. For similar reasons, microscopic examination of tissue is rarely conducted in acute toxicity studies unless there is some scientific reason to expect it would be useful.

The results of a well-designed acute toxicity study can help to predict likely target organ systems and the possible outcome in the event of massive human overexposure and in establishing risk categories for EPA or Department of Transportation classification, it can also help in dose selection for the initial repeated-dose toxicity tests to be conducted. An example of an acute toxicity study design is in Table 2.11.

Short-Term Toxicity Studies

The objective of short-term or subchronic toxicity studies is to describe and define the toxicity associated with repeated administration of high, but generally survivable doses of a test substance. This may include identification of target organs and systems, definition of the maximum survivable repeated dose, and the highest "clean" or no-effect dose. Short-term repeated-dose studies also serve as dose range–finding studies for longer-term repeated-dose studies.

Short-term toxicity studies range in duration of dosing from about 7 to 90 days. Mice typically receive a single daily dose of the test substance, 7 days per week, by the expected clinical route of administration. If the test substance is administered in the diet (or rarely, the drinking water), that admixture is available continuously. Short-term studies usually include three to four groups of mice exposed to different dose levels of the test substance and an additional group exposed to the carrier

Table 2.11 Typical Acute Toxicity Study Design for Mice

Number of mice/sex/dose group	3–5
Number of dose groups	1–3
Number of control groups	None
Dosing frequency	Single dose
Dosing days	1 day
Survival checks	Not done (part of *Clin. Obs.*)
Clinical observations	4 or more on the day of treatment, then 1–2 daily
Physical examinations	Not done
Body weights	Prior to dosing
Feed consumption	Not done
Number of reversal mice	None
Duration of reversal period	Not applicable
Blood collection	Not done
Hematology parameters	Not done
Clinical chemistry parameters	Not done
Urine collection	Not done
Necropsy	Gross (increasingly, but rarely useful)
Tissue collection	Rarely (specific cause only)

to serve as a control for the effects of treatment. Group sizes for these studies are on the order of 5–10 mice/sex/dose. Ideally, dose levels should be selected for these studies such that a few animals die at the highest dose prior to the completion of dosing (to assure exposure to the maximum survivable dose), and all survive at the lowest dose with minimal evidence of toxic effects. The middle dose or doses should be set at approximately equal log increments between the high and low doses. It is important to begin to identify the highest dose level that is free of serious toxic effects to determine whether the test substance is likely to be toxic to humans at the expected therapeutic dose or exposure level.

Parameters monitored in a typical short-term repeated-dose study may include daily observations for clinical signs of toxicity and mortality, weekly physical examinations, body weight and feed consumption, and terminal measurement of serum glucose and urea concentrations, serum aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase activity. Animals found dead or killed by design are typically submitted for gross necropsy, and selected tissues, such as adrenal gland, bone (sternum, including marrow), brain, heart, kidney, liver, lung, testis, and thymus, are collected, weighed (except for bone and lung), and processed for routine microscopic examination by a qualified veterinary pathologist. An example of a short-term toxicity study design is in Table 2.12.

Chronic Toxicity Studies (26 Weeks to 2 Years)

The objective of chronic, or long-term, toxicity studies is to refine the description of the toxicity associated with long-term administration of high survivable doses of a test substance. Chronic toxicity studies are more commonly conducted in rats than in mice, but such studies can be conducted in mice, and this discussion describes objectives and practices for conducting such studies. Target organs and systems have usually been identified prior to the conduct of chronic studies, but it is the chronic studies that provide the best opportunities to understand the subtle changes associated with long-term administration of high doses and to focus more closely on the highest "clean" or no-effect dose. Chronic toxicity studies also serve to refine the doses to be administered in the carcinogenicity studies that typically follow them.

Table 2.12   Typical Short-Term Toxicity Study Design for Mice

Number of mice/sex/dose group	5–40
Number of dose groups	3–4
Number of control groups	1
Dosing frequency	Once, daily
Dosing days	Daily for 7–90 days
Survival checks	1–2 daily
Clinical observations	Daily
Physical examinations	Weekly
Body weights	Weekly
Feed consumption	Weekly
Number of reversal mice	None
Duration of reversal period	None
Blood collection	Terminal, all animals
Hematology parameters	None
Clinical chemistry parameters	Limited
Urine collection	Not done
Necropsy	Gross, all animals
Tissue collection	Limited list, all animals

Chronic, or long-term, toxicity studies range in duration of dosing from about 26 weeks to as long as 2 years, but most do not exceed 1 year. Single daily doses of the test substance are administered by the expected clinical route of administration. If the substance is intended for oral administration, the convenience and economy of administration in the diet (or rarely the water) become important. Diet admixtures are made available *ad libitum* unless they must be removed for a specific procedure during the study. Chronic studies usually include three groups of mice exposed to different dose levels of the test substance and an additional group exposed to the carrier to serve as a control for the effects of treatment. Chronic toxicity studies often include "reversal groups," or subsets of each dose group that are not sacrificed immediately upon the completion of treatment. The purpose of the reversal groups is to determine whether any toxic effects associated with treatment are permanent or subject to recovery or reversal. Mice in the reversal groups may be allowed from 2 to 4 weeks of recovery time from the end of treatment until necropsy. Group sizes for chronic studies are on the order of 20–50 mice/sex/dose. Sizes of the reversal groups, if they are included, may be about 25%–35% of the original dose groups. Dose levels should be selected for these studies such that there is substantial toxicity at the highest dose, but few if any treatment-related deaths. The low dose in chronic studies should confirm or, if necessary, refine previous estimates of the highest dose level that is free of serious toxic effects and thereby reinforce previous estimates of the relative safety of the test substance at the expected human dose or exposure level. The middle dose should be the approximate geometric mean of the high and low doses.

Parameters monitored in a typical chronic toxicity study may include daily observation for moribundity and mortality, weekly physical examinations, body weight and feed consumption, and terminal measurement of serum glucose and urea concentrations, serum aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase activity. Hematological parameters for mice are often limited to differential smear evaluations. While red blood cell counts, white blood cell counts, and hemoglobin concentrations can be determined, historically the values for mice were somewhat variable from many of the previously used laboratory instruments, so these parameters were not always evaluated. This has changed in the last five years. Animals found dead or killed by design are typically submitted for gross necropsy, and about 30–50 selected tissues are collected, weighed (except for bone and lung), and processed

Table 2.13   Typical Chronic Toxicity Study Design for Mice

Number of mice/sex/dose group	20–50
Number of dose groups	3
Number of control groups	1
Dosing frequency	Once, daily
Dosing days	Daily 26–52 weeks
Survival checks	Daily
Clinical observations	Not done
Physical examinations	Weekly
Body weights	Weekly
Feed consumption	Weekly
Number of reversal mice	25%–35% of main groups
Duration of reversal period	2–4 weeks
Blood collection	Terminal, all animals
Hematology parameters	Dif. smear, RBC, WBC
Clinical chemistry parameters	Limited list
Urine collection	Not done
Necropsy	Gross, all animals
Tissue collection	Comprehensive list, all animals

for routine microscopic examination by a qualified veterinary pathologist. An example of a chronic toxicity study design is in Table 2.13.

Carcinogenicity Studies (18–24 Months)

The objective of carcinogenicity studies is to determine whether the test substance is a carcinogen when administered at maximum tolerable doses for a period of time approaching the life expectancy of the mouse.

This objective is simpler in many respects than the objective of the longer toxicity studies. It is assumed that by the time carcinogenicity studies are undertaken, the chronic toxicity studies have been essentially completed, and the actual toxicity of the substance is about as well understood as it can be based on animal studies. Carcinogenicity studies are not usually encumbered by tests to further the understanding of toxicity, but rather are focused to maximize the ability to answer the single question of carcinogenicity.

Carcinogenicity studies in mice are generally designed to expose the animals for a period of 18 months to 2 years. Improvements in animal husbandry during the past decade have increased the life expectancy of most strains of mice, so there has been a tendency to extend carcinogenicity studies in mice to 2 years. The study design normally contains provisions to allow for termination of the study prior to the intended endpoint if excessive mortality is encountered. This provision is intended to ensure that an adequate number of survivors are sacrificed with a successful collection of all necessary tissues for a meaningful statistical analysis of tumor incidence. Carcinogenicity studies usually include three groups of mice exposed to different dose levels of the test substance and an additional group exposed to the carrier to serve as a control for the effects of treatment. Group sizes for these studies are on the order of 50–70 mice/sex/dose.

The high dose in carcinogenicity studies should be the maximum tolerated dose. This dose should produce evident toxicity by the end of dosing. A commonly held minimum criterion for evident toxicity is a decrement in body weight or body weight gain of at least 10% from the control to the high-dose group. If the test substance is not very toxic, most regulatory agencies will accept a carcinogenicity study in which the high dose is the highest dose that can be practically

Table 2.14   Typical Carcinogenicity Study Design for Mice

Number of control groups	2
Dosing frequency	Once, daily
Dosing days	Daily for 18–24 months
Survival checks	Daily
Clinical observations	Not done
Physical examinations	Monthly during first year, 2×/month thereafter
Body weights	Weekly during first 2 months, 2×/month thereafter
Feed consumption	Weekly during first 2 months, 2×/month thereafter
Blood collection	Optional periodic, terminal
Hematology parameters	Periodic peripheral smears, terminal smears, RBC, WBC
Clinical chemistry parameters	Not done
Necropsy	Gross, all animals (including those found dead during study)

administered, even though that dose does not produce evident toxicity. In this example, the animal has, in effect, "tolerated" the highest dose that could be administered.

The selection of lower doses is not as critical as in chronic studies because the concept of a "clean dose" of a known carcinogen is not widely accepted and will be of little value in attempting to commercialize a product.

The middle dose may become important if the high dose has been inadvertently set too high, resulting in excessive toxicity and early mortality. In that case, a middle dose that elicits evident toxicity without excessive mortality may become an acceptable maximum tolerated dose and may effectively "salvage" the study. As in chronic studies, the middle dose should be at the approximate geometric mean of the high and low doses.

The most important data generated in a carcinogenicity study are the histopathology data. Of particular importance are the data on the incidence of various types of malignancies in the different treatment groups. Control or even untreated mice normally will have some "background" incidence of various types of malignancies over the course of a carcinogenicity study. The key question then is whether the treated groups have a significantly higher incidence of "normally expected" tumor types than the control group, whether they have occurrences of "nonnormal" tumor types that are not seen (concurrently or historically) in control animals, and, most importantly, whether such incidence is attributable to the test substance.

Other parameters monitored in a typical carcinogenicity study may include daily observation for survival and moribundity, periodic physical examinations, periodic examinations for palpable masses, body weight, and feed consumption (especially important in dietary admix studies), and for some studies periodic peripheral blood smears and terminal red and white blood cell counts. Animals found dead or killed by design are submitted for gross necropsy, and a comprehensive list of 40–50 prescribed tissues plus any tissue masses, suspected tumors, and an identifiable regional lymph tissue are collected from each animal to be processed and examined histologically by a qualified veterinary pathologist for the evidence of carcinogenicity. An example of a carcinogenicity study design is in Table 2.14.

Teratology Studies

Mice are occasionally used in teratology studies to assess the effects of test substances on congenital defects in the young when administered to pregnant females. Mice have a regular, short estrus cycle, a short gestation period, high fertility, and typically produce relatively large litters of young. Mice rank behind rats and rabbits as the species of choice for assessing teratology, but there may be good scientific reasons to use mice in some instances. The conduct of Segment II teratology

studies in mice and rabbits with Segment I and III studies in rats provides an opportunity to evaluate teratogenicity in three species. While there is an advantage in conducting teratology studies in at least two species, preferably one of which is a non-rodent, some substances (e.g., certain antibiotics) are especially toxic to rabbits, making teratology testing impractical in that species. In that case, mice become the second best species available (after rats) for teratology testing and are usually the choice as the second species. A significant disadvantage in using mice for teratology studies is that they are much more cannibalistic than rats or rabbits. This characteristic renders the species unusable for Segment I and III studies, in which pregnant females are allowed to deliver their young, and limits them to use in Segment II studies, in which the young are delivered by caesarean section on gestation day 18, prior to delivery. Mating can be confirmed in mice by daily inspection of cohabitating females for the presence of a vaginal plug. The copulatory plug in mice is much more persistent than in rats, in which mating must be confirmed by vaginal lavage and microscopic examination.

Segment II Teratology Studies

A Segment II teratology study is conducted to assess the effects of a test substance on fetal survival and congenital malformations (teratology). Females are mated and monitored daily for the presence of copulatory plugs. The presence of a plug confirms that mating has occurred, and the day of discovery is defined as gestation day 1. Mated females are dosed with the test substance from gestation days 6 through 15, a period that begins soon after implantation (day 5) and continues through completion of organogenesis (day 13). This dose period exposes the young throughout the period of organogenesis, but tends to minimize preimplantation embryotoxicity and postorganogenesis maternal and fetal toxicity. Young are delivered by caesarean section on gestation day 18, prior to normal parturition on day 19, to avoid cannibalism. The maternal reproductive organs are examined for numbers of corpora lutea, implantations, resorptions, and live and dead fetuses. The fetuses are weighed, sexed, and examined for gross, visceral, and skeletal abnormalities. An example of a Segment H teratology study design is in Table 2.15.

Genetic Toxicity Studies

The objective of genetic toxicity testing is to identify and describe the effects of agents that specifically produce genetic alterations at subtoxic doses. Mice are used in a variety of genetic toxicity

Table 2.15   Typical Segment II Teratology Study Design for Mice

Number of female mice/dose group	20
Number of dose groups	3
Number of control groups	1
Dosing frequency	Once, daily
Dosing days	Days 6–15 of gestation
Survival checks	Daily
Clinical observations	Daily
Physical examinations	Not done
Body weights	1–3 ×/week
Feed consumption	Not done
Number of reversal mice	None
Duration of reversal period	Not done
Blood collection	Not done
Caesarean section	Day 18 of gestation
Necropsy	All dams gross, all fetuses external, and 113 of fetuses visceral exam at caesarean section

study designs in an effort to achieve this objective. Neither a comprehensive listing of genetic toxicity procedures using mice nor a comprehensive description of any number of those procedures is within the scope of this chapter. Rather, a few of the most commonly employed procedures will be summarized. The reader is referred to other sources such as Brusick (1980), Thorgeirsson (1982), and Dean (1983, 1984) for more detailed discussions of genetic toxicity and more comprehensive descriptions of some of the specific tests used in that field. Kaput (2005) reviews the interaction between nutrition and genomics.

Mouse Micronucleus Assay

The objective of the mouse micronucleus assay is to determine whether a test article causes disruption and separation or breakage of chromosomes. The mouse micronucleus assay is one of the most commonly conducted *in vivo* tests for genetic toxicity. Comparison of the incidence of micronuclei in proliferating cells from treated versus control mice provides an indirect measurement of chromosome damage in somatic cells. Micronuclei can be formed only as a result of disruption and separation or breakage of chromosomes, followed by cell division. The preferred cells for evaluation are newly formed erythrocytes in mouse bone marrow because the micronuclei formed in these cells are not expelled during the last division in which the nucleus is extruded from the normoblast.

One or more dose levels of test article and a control treatment are administered to separate groups of at least five mice per sex. The highest dose should be the maximum tolerated dose or one that produces some evidence of cytotoxicity. It is important that high-quality (e.g., specific pathogen-free [SPF]) mice of known genetic stability and consistent species, strain, source, age, weight, and clinical condition be used to assure comparability with historical controls. Each animal typically receives a single dose of test or control article. Bone marrow samples are collected at a minimum of three different intervals after dosing, ranging from 12 to 72 hours. At least 1000 polychromatic erythrocytes are evaluated for the presence of micronuclei for each test and control mouse. An example of a mouse micronucleus assay study design is in Table 2.16.

Heritable Translocation Assay

The objective of the heritable translocation assay (HTA) is to assess the potential of a test article to induce reciprocal translocations between chromosomes in germ cells of treated male mice. This assay has the advantage of detecting transmissible genetic alterations, which are potentially more damaging to the gene pool than nontransmissible or lethal changes. Induced translocations can be detected by mating the F1 progeny of treated males with untreated, unrelated females. Translocations will be evidenced by a reduction in the number of viable fetuses sired by

Table 2.16   Typical Mouse Micronucleus Assay Study Design

Number of mice/sex/dose group	5 or more
Number of dose groups	1 or more
Number of control groups	2, one positive, one negative
Dosing frequency	Single dose
Dosing days	One day
Survival checks	Daily
Clinical observations	Not done
Body weights	Prior to dosing
Feed consumption	Not done
Bone marrow collection	3 or more intervals, from 12 to 72 hour after dosing
Polychromatic erythrocytes evaluated/mouse	1000 minimum

Table 2.17   Typical Mouse Heritable Translocation Assay Study Design

Number of male/mice/dose group	30
Number of dose groups	3
Number of control groups	1–2 (negative; pos. optional)
Dosing frequency	Once daily
Dosing days	Daily, 7 weeks
Survival checks	Daily
Clinical observations	Not done
Mating of dosed males	Upon completion of dosing
Number of F, males mated/group	200
Number of F is examined cytogenetically for translocations	All sterile and semisterile
Tissue collection	Testes

affected males. The presence of translocation figures in meiotic metaphase serves as cytogenetic verification of the presence of reciprocal translocations.

The HTA typically consists of three groups of 30 male mice each, treated with different dose levels of the test article, and a negative control group treated with the dosing vehicle. A positive control group is optional. As the period of spermatogenesis in the mouse is about 7 weeks, all treated and control animals are dosed on a daily basis for 7 weeks. The dosing route is usually oral gavage, and the dose levels are selected on the basis of the oral LD50. The high dose is typically one-eighth of the LD50, the medium and low doses about one-third and one-tenth of the high dose, respectively. Upon completion of dosing, each male is mated to two females. Two hundred healthy males are selected from the offspring of each of these groups of matings and allowed to reach sexual maturity, whereupon each male is mated to three virgin females. The females from this second mating are sacrificed about 3 weeks after cohabitation with the males was initiated, and the number of living fetuses and resorbing embryos present in the uteri are counted. Any male that produces 10 or more living fetuses in any one female is considered fertile, and no further matings are needed. Males falling below that criterion are mated to an additional set of three females, and the evaluation is repeated. Failing males may be remated up to three times. Males that never succeed in producing at least one litter of 10 or more living fetuses are considered sterile (or semisterile), are sacrificed, and their gonadal cells are examined for cytogenetic evidence of translocations. An example of an HTA study design is in Table 2.17.

Microbial Host-Mediated Assay

The objective of the microbial host-mediated assay is to determine the ability of a mammalian system (e.g., the mouse) to metabolically activate or detoxify a test article with respect to its mutagenic potential. The mutagenic potential is measured by means of one of a variety of microorganisms, depending on the specific types of mutations being investigated (e.g., base-pair substitution, frame shift). Some of the microorganisms used for this type of testing include various strains of *Salmonella typhimurium*, *Escherichia coli*, and *Neurospora crassa*. The results of the host-mediated assay can be compared with the direct effect of the test article on the same tester strain of microorganisms to determine whether the host (e.g., mouse) is metabolically activating, detoxifying, or having no effect on the mutagenic potential of the test article. The microbial host-mediated assay, then, is an attempt to combine the convenience of microorganisms for detecting hereditary changes with the metabolism of the test article gained by administration to a whole animal.

The microbial host-mediated assay consists of three groups of about 10 mice each, treated with different dose levels of the test article, a negative control group treated with the dosing vehicle, and a positive control group. Doses are administered daily for up to about 5 days. The high dose is usually

Table 2.18   Typical Mouse Microbial Host-Mediated Assay

Number of mice/dose group	10
Number of dose groups	3
Number of control groups	2 (one positive, one negative)
Dosing frequency	Once, daily
Dosing days	Daily for 5 days
Survival checks	Daily
Clinical observations	Not done
Body weights	Prior to dosing
Tester organism administered	After last dose
Tester organism recovered	e.g., 1, 2, and 4 hours after administration
Necropsy	Not done
Tissue collection	Liver or other appropriate tissue for organism recovery

about one-half of the LD50, with the medium and low doses about one-third and one-tenth of the high dose, respectively. The preferred route of administration is oral, but intramuscular (im) or ip injection may be used if necessary. At the end of the period of test article administration, the tester strain of microorganism is administered, usually intravenously. Following appropriate incubation periods (e.g., 1, 2, and 4 hours), mice are sacrificed, and the microorganisms are collected, frequently from liver tissue. The collected microorganisms are grown on minimal agar plates to assess mutation rate. An example of a microbial host-mediated assay study design is in Table 2.18.

Special Studies

The diversity of toxicity study designs using the mouse as a test system to examine a specific hypothesis defies description. Many of these designs do not strictly fit within the major section headings chosen for this chapter and might be referred to as "special studies." This chapter is intended to focus on the more commonly conducted types of toxicity studies in mice, but it might be useful to describe two special study designs here as examples of some of the interesting endpoints that can be evaluated in this species.

Mouse Ear Swelling Test

The concept of a mouse ear swelling test (MEST) is that sensitization can be detected by measuring edema in the ear of a mouse that results from topical application of a test article to an animal that has been previously sensitized by means of dermal application to the abdomen.

The objective of MEST is to provide an alternative test for dermal sensitization potential that makes more efficient use of animals, labor, and other resources than traditional study designs conducted in guinea pigs. A MEST is often preceded by a dose-finding activity to identify the highest concentration of test article that is no more than minimally irritating to the abdominal skin and the highest concentration that is nonirritating to the ear. These concentrations are then employed in the sensitization assay.

The sensitization assay is carried out in three phases: an induction phase, a challenge phase, and if necessary, a rechallenge phase. The sensitization assay requires 15 mice treated with the test article and 10 treated with a control substance, typically the vehicle used for the test article. During the induction phase (study day 1), the abdomen is shaved using a small animal clipper, and 20 µL of 1:1 emulsion of Freund's complete adjuvant in distilled water is injected intradermally at each of two abdominal sites on opposite sides of the ventral midline. Once daily on study days 1, 2, 3, and 4, the stratum corneum is stripped from the abdominal skin using adhesive tape, and the appropriate concentration of test article is applied to the abdomen at a volume of 100 µL.

Table 2.19 Typical Mouse Ear Swelling Test Study Design

Number of Male/Mice/Dose Group	15/Treated Group, 5/Control Group
Number of dose groups	1
Number of control groups	2
Dosing frequency	Once daily
Dosing days	Days 1, 2, 3, and 4 (induction)
	Day 2 (challenge)
	Day 18 (optional rechallenge)
Survival checks	Daily
Clinical observations	Not done
Body weights	Not done
Ear thickness measured	Days 10, 12, and 13 (challenge)
	Days 17, 19, and 20 (rechallenge)
Necropsy	Not done
Tissue collection	None

On study day 11, the challenge phase is initiated. The appropriate concentration of test article is applied to the skin of the left ears of all treated and 5 of the 10 control mice at a volume of 10 μL, and an equal volume of the control (vehicle) is applied to the right ears of those same animals. Thicknesses of both ears of all treated and the five selected control mice are measured on days 12 and 13. Any mouse with a left ear thickness ~120% of its right ear thickness is considered to be a positive responder. If one or more mice are judged positive in the absence of primary irritation (any control mice with left ear thickness >110% of right ear thickness) on study day 12 or 13, the test article is considered to be a sensitizer. Evidence of primary irritation requires that the test be repeated using a lower concentration for the challenge dose.

If results are negative (all mice with a left ear thickness increase of <10%) or equivocal (some mice with increases of 10%–19%, but none >20%), a rechallenge is conducted on study days 17 through 20. Baseline ear thicknesses are measured on study day 17. The test article is applied to the right ears of all test mice and the control mice that were not used during the initial challenge on study day 18. Ear thicknesses of the right ears of all test mice and the new control mice are measured on study days 19 and 20, and are compared to the baseline thicknesses taken on day 17. The criteria for positive response are the same as for the initial challenge. An example of a MEST study design is in Table 2.19.

Dermal Carcinogenicity (Skin Painting) Study

The concept of the dermal carcinogenicity (or skin painting) study is that carcinogens, or of more recent interest, cocarcinogens and tumor promoters can be evaluated or their potencies compared in as little as a few months of testing.

The carcinogenicity of some chemicals (e.g., polycyclic aromatic hydrocarbons) can be detected easily by the production of papillomas or carcinomas. Mouse skin apparently functions in this system because it contains enzymes necessary to produce the active intermediates that lead to initiation. Tars from tobacco, coal, and various petroleum products show active carcinogenic potential in this system, although many of the same products are not carcinogenic when administered systemically. Hepatic detoxification of systemically administered doses may account for this difference.

This study design has been especially useful in recent years in the study of cocarcinogens and tumor promoters. In a typical study design, groups of 25–50 mice might receive from one to a few systemic doses of a known tumor initiator. Following receipt of the initiator, the fur over the anterior portion of the back is shaved, and the suspected tumor initiators are applied to the skin of the back at

a frequency of two to three times per week. Shaving will need to be repeated approximately weekly. Development of papillomas or carcinomas of the skin is readily visible in the shaved area. Active chemicals are often detected within a few months' treatment. This study is generally intended to continue treatment for a period of about 30–40 weeks, but may be continued for up to 2 years if necessary.

Obvious advantages of this study design include its relative efficiency in terms of animal numbers and labor, and its relative brevity compared to a conventional 18- to 24-month carcinogenicity bioassay. It is reasonable to conclude that positive findings of carcinogenicity in this test would make a conventional carcinogenicity bioassay unnecessary. Negative findings in a dermal carcinogenicity test, however, would not assure the absence of carcinogenic potential, and a conventional bioassay would still be necessary.

Disadvantages of this procedure include difficulty in accurate quantification of dose, as the topically applied dose can run off the animal, be scratched or licked off, or can accumulate as a crust, effectively reducing absorption. Another criticism centers around the fact that if treatment with a suspected promoter is interrupted after a period of 60 days or so, evident papillomas often regress, raising the question of whether they represented sites of true carcinogenicity.

Dosing Techniques

Techniques are available to administer test substances to mice by most routes of potential human exposure. The choice of a route of administration for a toxicity study should consider the expected route of human exposure and any other scientific objectives that need to be achieved to facilitate safe use of the test substance. Of the various routes available, most test articles will have the most rapid onset of effects and the greatest potency when administered by the iv route, followed in approximately descending order by the inhalation, ip, sc, im, intradermal (id), oral, and topical routes (Klaassen and Doull, 1980). The expected route of human exposure is probably the most important single determinant of route for toxicity testing. In the discussion that follows, the most commonly employed routes for toxicity testing in mice, oral dosing, will be discussed first, followed by the less commonly employed routes.

Oral Administration

Oral administration is probably the most frequently used route of exposure for toxicity testing in mice. Many products are intended for oral administration to humans, and many others are subject to accidental ingestion. Oral administration subjects the test substance to limitations of absorption and metabolism that are similar but not necessarily identical to those in humans. Mice, like rats, differ from many other species in that they do not have an emetic response. For this reason, large doses of substances that would cause emesis in dogs or primates will be retained in the stomachs of mice. While this characteristic facilitates testing at high doses and maximizes potential exposure to toxic effects, it may lead to an overestimate of potential human toxicity because the animal lacks the protective aspect of the emetic response. Another area in which mice differ from dogs and primates is that mice are nocturnal. This characteristic adds some pharmacokinetic variables to the equation for extrapolating toxicity findings from the mouse to the human. Doses that are administered during the day are administered to animals that are in the lower phase of their circadian metabolic cycles. This may mean slower absorption, slower metabolism to either more or less toxic metabolites, and/or slower elimination of the test substance. Conversely, test substances administered in the diet or drinking water will be largely consumed at night, as that is when mice consume most of their daily intake of food and water. While this regimen more closely approximates human consumption during the active part of the day, it makes observation of the animals during the period of peak exposure and metabolism difficult. Three means of oral administration are oral gavage, dietary admixtures, and mixture with the drinking water.

Gavage

Oral gavage offers advantages of precisely measured doses that can be administered at precise times. Doses can be administered during the day so animals can be conveniently observed for toxic effects during the first few hours after dosing. Volatile substances and those that lack stability over longer periods in the presence of diet, air, or water can be effectively administered by this method. Gavage allows administration of unpalatable substances that might not be accepted in the diet or water. There are disadvantages associated with gavage administration. The test substance either must be a liquid or must be soluble or suspendable in a liquid vehicle system. The method is relatively labor intensive compared to diet admix. The processes of daily handling and intubation of all animals engender the risk of injury during the intubation process, including esophageal puncture and aspiration of test article. In addition, the process of frequent handling causes stress to the animals. While it is convenient to administer doses during the day, daytime is the period of lowest metabolic activity for nocturnal species such as mice. This circadian effect may not be most representative of diurnal species such as humans.

Gavage administration entails intubation of the mouse with an intubation needle attached to a graduated hypodermic syringe. The dose is administered into the esophagus. Intubation needles for mice are typically constructed of stainless steel tubing with a stainless steel ball tip to reduce the probability of esophageal perforation and reflux and aspiration of the dose. Acceptable tubing sizes range from 22 to 18-gauge, with the larger bore reserved for older mice (e.g., $\geq$25 g). Tubing length is not critical but may range from 1 to 3 in. The ball tip is typically 1.25–2.25 mm in diameter. Intubation needles are available commercially (e.g., Popper & Sons, Inc., New Hyde Park, New York) in straight and curved configurations. The choice of shape is a question of personal preference on the part of the dosing technician. Prior to dosing, the test substance must be prepared in a liquid form at an appropriate concentration. Liquid test substances may require dilution. Solid substances will require either dissolution or suspension in an innocuous vehicle. The preferred vehicle is water. If the substance is insoluble in water, various agents may be added to improve wetting (e.g., 0.1% v/v polysorbate 80) and to reduce settling (e.g., 0.5% w/v methylcellulose). Suspensions should be analyzed prior to administration to assure proper concentration, homogeneity, and stability of the substance in the suspending vehicle. Appropriate dose volumes for gavage administration are in the range of 5–10 mL/kg of body weight, but volumes as high as 20 mL/kg can be administered carefully, particularly in acute studies in which the mice have been fasted prior to dosing.

For the actual process of dose administration, the mouse should be weighed, and the individual dose calculated. The appropriate dose volume should be drawn into the dosing syringe, and any air bubbles should be expelled. The mouse is then picked up by the skin of the back and neck, and the head tipped back to form a straight line from the nose through the back of the throat and to the stomach.

The intubation needle is inserted to the back of the mouth, then gently tipped back, if necessary, to enter the esophagus. The mouse will usually facilitate entry into the esophagus by swallowing the ball of the needle. One successful approach is to envision the tip of the sternum as a "target" for the tip of the intubation needle. When properly positioned, the tube can easily be inserted to a reasonable depth, but it need not reach the stomach. When in position, the dose should be administered slowly to avoid reflux, but promptly enough to reduce the likelihood that the mouse will struggle and injure itself.

Dietary Admixtures

Oral administration by dietary admixtures offers several advantages, including ease of administration, minimal handling of animals for dosing, and elimination of the risk of injury associated with intubation. The method offers relatively precise dose administration for the group (better than water mix; not as good as gavage), as both mean food consumption and mean body weight for

periods of a week or longer are easily measured. Dry insoluble substances can be administered easily, and administration of test substances to mice during the "awake" phase of their circadian cycle is an advantage. There are disadvantages associated with dietary admix. Accuracy of individual doses is lower than with gavage, and there is not a single identifiable time of dosing. Volatile substances and those that lack stability over periods of at least 4–7 days in the presence of diet, air, or water are precluded from this method. Diet admixtures must be sampled, and the samples analyzed periodically to assure proper concentration and homogeneity of the mixture during the course of the study. Unpalatable test substances typically result in reduced dietary intake, which leads to an increase in the concentration of test substance in diet during subsequent weeks in an attempt to reachieve the desired doses. The increased concentrations may be even less palatable, leading to further reduction in dietary intake and, in some cases, eventually to malnutrition. In any dietary study, results should be evaluated carefully to discriminate between changes associated with altered nutritional status and true test substance toxicity.

Oral administration by dietary admix entails presentation of a mixture of the test substance in diet in place of the normal diet received by the animals. The concentration of test article in diet is adjusted, based upon the most recently collected data on mean body weight and food consumption for each sex and dose group, to provide the desired doses of test article during the period in question. Early in a study, when body weight and food consumption are changing due to rapid growth of the animals, projections of the mean body weight and food consumption for the coming period should be based upon both the most recent measurements and the rate of change (slope of the plot) of those parameters over several recent periods. If test article stability permits, a convenient period for the measurement of body weight and food consumption is about 1 week.

Test substances are usually mixed with the meal form of rodent diet in one or more of a variety of mechanical blenders. Common types of blenders include the Turbula®, paddle style, and twin shell or "Y" blenders. Turbula and paddle blenders are illustrated in Figures 2.2 and 2.3. When large quantities of diet admix are required, it may be advantageous to blend the test substance with the diet in two steps, preparing a premix of 1–2 kg in a blender such as a Turbula, then adding the premix to a larger-scale blender, such as a paddle blender. This procedure often produces a more homogeneous mix in a shorter blending period.

Following mixing, the appropriate concentration of blend is dispensed into animal feeders for presentation to the mice. Feeders are weighed when they are placed in the cages and when they are removed to determine the average amount of feed consumed per day. Mice are weighed at the same
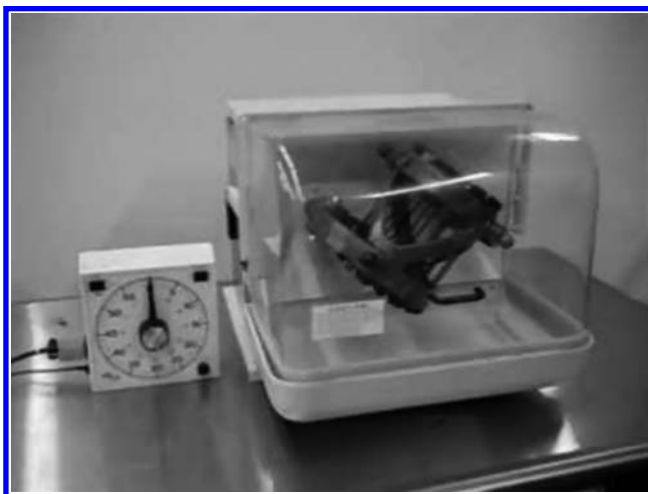
Figure 2.2   Small volume dietary mixer.
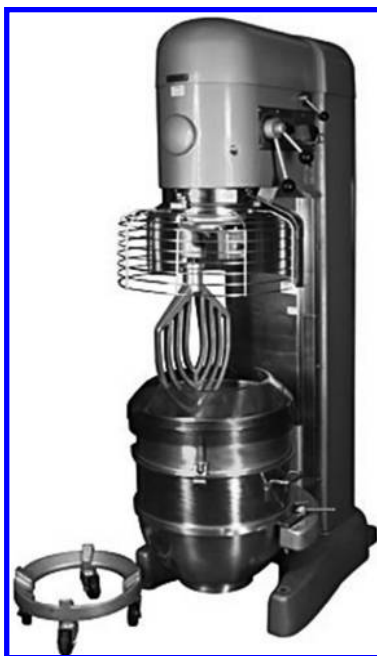
Figure 2.3 Bench top diet mixture (not enclosed).

time, both to determine body weight gain over the period and to calculate the food consumption in grams per kilogram of body weight per day. Based upon this calculation, the concentration of test substance in diet may be varied up or down to more closely approach the intended doses of test substance in milligrams per kilograms per day.

Among potential problems that may be encountered in conducting dietary admix studies, two may lead to inappropriate calculations of feed consumption, leading to incorrect calculation of concentrations for future periods. These problems are excessive feed spillage, which may result from mice digging or playing in their feeders, and contamination of the feeder with urine and feces, which may result from mice living in their feeders. Feeders should be checked daily for excessive spillage at the time survival checks and/or observations are conducted, and excessive spillage should be documented. Contamination with urine and feces will lead to an incorrectly high feeder weight at the end of the feed consumption period and an underestimate of true feed consumption.

Excessive contamination should be documented. Animals with excessive spillage and those with excessive contamination of feeders should be excluded from the calculation of mean body weight and food consumption used to prepare future concentrations of diet admix.

Drinking Water

Administration of a water-soluble test substance in the drinking water has many of the same advantages as administration of a dietary admix. This method is rarely used for toxicity studies, however, because of the difficulty in accurately measuring the quantity of water actually consumed. While graduated water bottles can be used, spillage due to mice rubbing against sipper tubes, inefficient drinking, evaporation, vibration, and leaking bottles makes these measurements imprecise. If that practical problem could be solved, administration in the drinking water would bear little conceptual difference from dietary admix. Stability of environmental temperature and humidity is essential to the conduct of a study in which the test article is mixed in the drinking water, as

increased temperature and/or lowered humidity lead to increased water consumption. Water consumption must remain relatively stable to allow the calculation of appropriate concentrations of test article in water to achieve study objectives.

From a practical standpoint, administration of test article in the drinking water has many similarities to administration by dietary admix. It is essential that the test article be both soluble and chemically stable in water for the period of presentation. That period should be in the range of about 2 days to a maximum of about 1 week. Water remaining in a water bottle for periods in excess of a week may become heavily contaminated with bacteria. Analogous to the situation with diet admix, the precision of dose administration is directly related to the accuracy with which average daily water consumption and body weight can be measured. Concentrations of test article in water should be adjusted, if necessary, after each measurement of average daily water consumption and body weight to assure precise dose administration.

As solubility in water is a prerequisite for this method, mixing procedures are usually simpler than for diet admixes. Homogeneity analysis should not be required for a true solution, but samples should be analyzed regularly to confirm that concentrations are what they were intended to be.

Water can be provided in graduated bottles. Contents of the bottles should be recorded at the beginning of the consumption period (but after the bottles are placed on the cages to accommodate spillage during that operation) and again at the end of the consumption period to determine the average daily water consumption. Evidence should be documented. Difficulty in accurately measuring the amount of water actually consumed by the mice is the largest disadvantage to this method of administration.

Intravenous Injection

Intravenous injection offers the advantages of immediate, complete systemic availability of a precise dose at a known point in time. The process of absorption is eliminated, as is the possibility that some or all of the test article may be metabolized by the liver prior to distribution to the systemic circulation and target organs. Most substances exhibit the biggest potency and rapidity of onset of activity of all routes of administration when administered intravenously. Intravenous administration provides a useful benchmark against which absorption and bioavailability from administration by other routes can be compared.

It is essential that test articles administered intravenously be in solution at the time of administration and remain in solution after injection. Solutions that are subject to precipitation by changes in pH, temperature, or osmolarity should be confirmed at physiological conditions to assure that they will not precipitate after injection. Introduction of insoluble particles, such as those in a suspension, introduces a high probability of embolism, particularly in the pulmonary capillary bed, which will produce severe moribundity or death. The toxicologist is left with the problem of differentiating such moribundity or mortality from the true toxicity of the test article.

Other characteristics of an iv solution that should be evaluated prior to undertaking an iv toxicity study involving repeated dosing include the potential for the solution to cause hemolysis or vascular or sc irritation. Hemolysis may be a result of the administration of solutions of inappropriate osmolarity (hypotonic solutions are particularly damaging), in which case the problem can be resolved by adjusting the osmolarity of the solution.

Ideally, solutions for iv administration should be isotonic to blood and have a pH of about 7. Usually, a pH in the range of about 5–9 will be acceptable. Solutions that cause appreciable vascular or sc irritation may result in sufficient injury to the veins and surrounding tissue to preclude repeated administration.

The rate of iv injection is an important variable that must be controlled fairly precisely to achieve reproducible results within a study. If the iv toxicity of two or more test articles is to be compared, it is essential that each be administered at the same rate. Intravenous injections can be administered

as a bolus over a period as short as a few seconds, as a continuous 24-hour/day infusion, or over just about any interval in between.

There is nothing particularly magical about any specific dosing period, but it is critical that it remains constant. As a practical matter, an injection period of about 2 min is a reasonable upper limit for handheld injections into caudal veins of reasonable numbers of mice. Longer periods lead to very slow injections, increase the risk of extravasation, are time consuming, and are difficult and tiring for the toxicologist. Injection periods much shorter than 1 min increase the likelihood that an inordinately high peak plasma concentration that may precede mixing with the total blood volume may compromise the survival of the animal. A corollary to the artificially high peak plasma concentration associated with a short injection period can occur if the injection is administered at an uneven rate. It is particularly critical that the rate of injection not be increased during the last half of the injection period, as this is a time when the animal has already received an initial "loading," and the deleterious effect of increasing the rate of administration will be amplified. In our laboratory, we have found that administration of volumes of 5–10 mL/kg body weight (0. 15–0.30 mL for a 30 g mouse) administered evenly over a period of 2 min represents a good compromise. One of the biggest disadvantages of iv administration is that it is a very labor-intensive procedure, requiring more time per animal than any other route. In addition, repeated daily administration to caudal veins of mice for periods longer than 2–4 weeks becomes technically difficult owing to the accumulation of scar tissue and occasional trauma.

Long duration or continuous 24-hour per day infusions, while possible, are technically difficult and will not be discussed in detail here. As a practical matter, long-duration injections or continuous infusions are typically administered through a surgically implanted catheter using an infusion pump. The catheter is usually placed in a large superficial vein such as the jugular or femoral vein, then exteriorized at a site such as between the scapulae, which is difficult for the animal to chew or scratch. The surgical procedure is relatively simple. The difficulty lies in keeping the cannula patent and secure in the vein during recovery after surgery, then through the period of dosing. The maximum volume of infusion, even over a continuous 24-hour period, should not exceed about 20–30 mL/kg/day, which will typically be less than 1 mL administered over a 24-hour period. This infusion rate is so slow as to be difficult to administer, even with a high-quality infusion pump. The catheter must be attached to the mouse in a way that prevents mutilation of the catheter by the animal without undue limitation of mobility.

Intravenous administration entails injection of the desired dose into an appropriate vein. In a typical study, mice will receive a single injection daily over a period of about 2 min or less. Such injections are usually administered into a lateral IMM (tail) vein, using a hypodermic needle attached to a graduated syringe. Hypodermic needles used for caudal vein injections in mice are usually no more than 1 in. long and in the range of about 23–25-gauge. The smallest hypodermic syringe that will contain full-dose volume will provide the greatest precision in dose measurement, but 1 cc disposable syringes are often used.

The needle should be installed on the syringe such that the bevel of the needle faces the side of the syringe that will be used to measure the dose. This will allow the graduations to be read easily when the needle has been inserted into the vein in an up configuration. Typical needles and syringes are shown in Figure 2.4. A stopwatch that is easy to read is useful for timing dose administration, and an electronic timer that is activated by a foot pedal is most convenient. A convenient restrainer should hold the mouse securely, but without undue risk of suffocation or injury, while allowing fire access to the tail. Examples of a useful restrainer, timer, and foot-activated switch are shown in Figure 2.5. A source of warm water and/or a tourniquet device is useful, as is a supply of small gauze sponges. A tourniquet that can be easily operated with one hand is pictured in Figure 2.6. This tourniquet can be constructed from a disposable plastic syringe and a piece of suture. The larger the syringe size, the more pressure it is able to exert on the suture loop. Relatively large (e.g., size 0) braided silk suture should be used for the loop to minimize the risk of cutting the skin

Figure 2.4   Disposable plastic syringe for intravenous dosing.

Figure 2.5   Complete equipment set for parenteral dosing of mice.
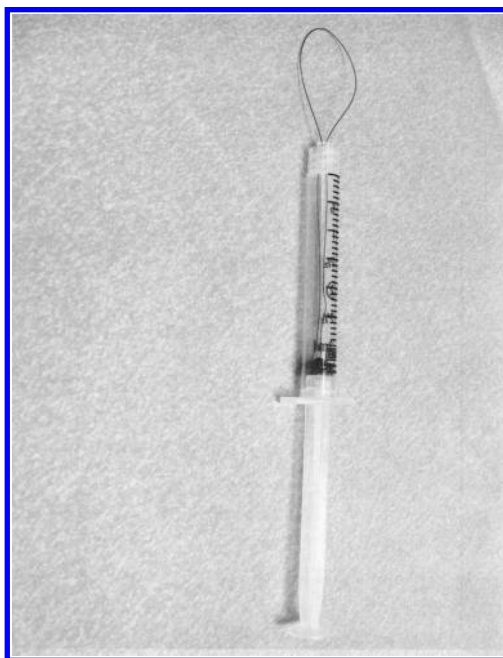
Figure 2.6 Syringe for tail vein dosing with suture loop attached to hold syringe in place.

of the tail. The suture loop is attached to the plunger inside the barrel of the syringe, then threaded out through the tip of the syringe.

Prior to dosing, the dosing solutions should be prepared. Solutions should be analyzed periodically to assure that proper concentrations are being attained. Each mouse should be weighed, and its dose calculated. As previously noted, doses in the range of 5–10 mL/kg are acceptable for injection over periods of about 30 s to 2 min. The mouse is placed into the restrainer, and the appropriate dose is drawn into the syringe. Any air bubbles should be expelled from the syringe. This is most important for iv injections. A tourniquet device can be applied at this point, but should not be applied too tightly. The objective is to block venous return, but not arterial supply, thus dilating the veins. As an alternative to a tourniquet, some toxicologists prefer to warm the tail with a gauze sponge wetted in warm (not hot) water to enhance vasodilation. The tail is now held in one hand, while the needle is inserted with the other. The needle should be inserted with the bevel up to minimize the chance of puncturing through both sides of the vein. Successful venipuncture will result in the reflux of a small amount of blood into the hub of the needle. Owing to the small volume of blood that usually refluxes, this phenomenon will be most easily visualized if needles are used that have transparent "flashback" hubs. The initial attempt at venipuncture should be made toward the tip of the tail, such that if the vein is missed, a subsequent attempt can be made closer to the base of the tail without risk that the dose will leak out of the initial hole. When the needle is securely positioned in the vein, it can be held with the "tail-holding hand" while the plunger of the tourniquet is depressed to open the vein with the "dosing hand."

Now that the needle is in the vein and the vein is open, the timer can be started, and the dose can be administered. One convenient method to assure even dose administration is to divide the dosing period and the dose volume into a convenient number of parts. A 2 min dosing period might be divided into eight 15 s intervals, and the dose volume divided by eight. The doser can then administer one-eighth of the total dose volume over each 15 s interval for 2 min to assure a relatively even rate of injection. When the full dose has been administered, a clean, dry gauze sponge should be

pinched over the injection site, and the needle should be withdrawn. Maintaining pressure on the site of the injection for 10–30 s after withdrawal of the needle is usually adequate to prevent bleeding. The mouse can now be removed from the restrainer. As iv injections typically result in a rapid onset of activity, it is often appropriate to observe a mouse for the first few minutes after dosing for clinical signs of toxicity.

Intraperitoneal Injection

The ip route of administration generally offers the second most rapid absorption of a test article among the parenteral routes, with systemic availability second only to iv injection. Rapid absorption is conferred by the large surface area of the lining of the peritoneal cavity and by the rich blood supply to that area.

Intraperitoneal administration leads to absorption primarily through the portal circulation. As a result, test articles that are metabolized by the liver are subjected to extensive (or even complete) metabolism prior to reaching systemic circulation and target organs, unless, of course, the target organ is the liver, in which case toxicity may even be amplified. Test articles that are excreted in the bile are similarly subject to elimination prior to reaching the systemic circulation and target organs. Water-insoluble mixture aqueous suspensions, for example, can be administered by the ip route. This may provide the opportunity for rapid systemic absorption of lipid-soluble or certain other test articles. Solutions or suspensions for ip injection should be adjusted to a pH in the range of about 5–9 to reduce the potential for irritation. Osmolarity of the dosing formulation is not critical, as it is for iv injection. Dose volumes for ip administration are in the range of 5–10 mL/kg/day, but volumes as high as 20 mL/kg/day are acceptable, particularly if the study is of limited duration, or if it is known that the test article will be absorbed by the ip route.

One of the most significant disadvantages of ip administration is the risk of peritonitis. Peritonitis can result from any of three primary causes: physical irritation caused by accumulation of a truly insoluble or irritating test article in the peritoneal cavity, introduction of exogenous microbiological contamination, or microbiological contamination resulting from injury to the gastrointestinal tract or urinary bladder. The potential for a test article to produce physical irritation or chemical peritonitis can be assessed in studies of one to a few days in duration. While physical or chemical peritonitis is the most frequently seen form of peritonitis in toxicity studies, it is still found with only a small percentage of test articles. Mice are relatively resistant to microbiological infection, so microbiological peritonitis is even less common than physical or chemical peritonitis. Peritonitis resulting from injury during the injection process is extremely rare when injections are administered by qualified toxicologists. There is a slight risk to the animals of physical injury to a major organ or vessel during the injection process, but again this is extremely rare in the hands of qualified dosers.

Intraperitoneal injections are administered into the peritoneal cavity using a hypodermic needle attached to a graduated syringe. Each mouse receives a single daily dose, administered as a bolus, for the duration of the toxicity study. The injection should be administered into the animal's lower right abdominal quadrant to minimize the risk of injury to the liver, spleen, and bladder. For initial training purposes in dosing by the ip route, it is useful to sacrifice a mouse, then the abdominal cavity is opened to expose the internal organs that may be susceptible to injury during the injection procedure. This will allow a novice to hold the animal in a dosing position and clearly visualize where the lobes of the liver, the spleen, and the urinary bladder will be and the area of less vulnerability between these organs. Hypodermic needles used for ip injections to mice need be no longer than about 5/8 in. and should be the smallest diameter that will allow easy injection of the dose volume to minimize the trauma to the abdominal wall with commensurate potential for leakage. Needles in the range of 23–25-gauge are appropriate for use with solutions and suspensions of low viscosity. Suspensions of high viscosity may require the use of needles

with a larger bore. Needles as large as 19–20-gauge can be used, but require great care to avoid injury and leakage of the test article from the injection site.

Prior to initiation of a toxicity study, dosing formulations should be prepared, and samples analyzed for concentration and homogeneity of suspensions, if appropriate. Each mouse should be weighed, and its dose calculated. The appropriate dose is then drawn into syringe, and air bubbles are expelled. The mouse is picked up with one hand and held with the ventral surface toward the doser. Movement of the animal's right hind leg should be restricted to limit interference with the needle during dosing. The needle should be inserted at an angle of about 15°–30° into the abdominal cavity to facilitate penetration of the abdominal wall.

The location should be to the right of the midline (to avoid the spleen) at a position about midway between the lower edge of the liver and the urinary bladder to a depth of about 1 cm (3/8 in.). Following insertion, the needle is withdrawn slightly, moved about, and the angle of insertion is reduced to assure that the tip has not penetrated or snagged any internal organs. The dose is now administered as a bolus, and the needle is withdrawn. If a large-bore needle has been used, it may be necessary to apply gentle finger pressure over the injection site for a few seconds to prevent leakage of the dose.

Intramuscular Injection

The im route of administration is less commonly used in toxicity testing, but it may be appropriate if the test article is intended for im administration to humans. The im route generally results in slower absorption of a test article, with lower peak plasma levels, but more sustained effects than iv or ip injection. The rate of absorption can be influenced by the amount of vascular perfusion of the tissue surrounding the injection, the vehicle, and the injected volume, which indirectly may alter the surface area of tissue available for absorption. Coadministration of a vasodilator generally increases the rate of absorption, whereas coadministration of a vasoconstrictor generally decreases that rate. Administration of the test article as a solution or suspension in a viscous poorly absorbed vehicle generally retards absorption. The ability to control the rate of absorption can be a significant advantage in some cases, as it allows the toxicologist to administer a dose of a test article that may be absorbed over a period of many hours or even days. This can be especially useful in the case of test articles that have short half-lives after absorption, as a result of rapid metabolism and/or elimination. Limitations to im dosing include the limited number of muscle groups in the mouse that are large enough to accept dosing, e.g., the muscles of the posterior aspect of the femoral region and the small dose volume that can be administered. If possible, the same injection sites should not be treated every day to allow time for absorption and recovery from the trauma of dosing. This means that while a single acute dose might be divided into the hind limbs, repeated daily doses should be administered into alternate limbs. The dose volume should not exceed 1.0 mL/kg per injection site, or about 0.03 mL for a 30 g mouse, and smaller volumes are preferable. An acute study in which each animal is dosed once would allow 1 mL/kg to be administered into each hind limb, for a total dose volume of 2 mL/kg. This dose volume coupled with the limit of solubility or suspendability of the test article in the vehicle selected may restrict the maximum dose of test article below toxic levels. A further limitation on toxicity testing by the im route is that the formulation to be injected must not cause significant local irritation, particularly if repeated doses will be administered. This limitation may require that a separate study be conducted to assess im irritation potential prior to initiation of a repeated-dose study by this route. Intramuscular injection is more labor intensive than most other routes with the exception of iv injection.

Intramuscular injections are administered into the large muscle groups of the posterior aspect of the femoral region using a hypodermic needle attached to a graduated syringe. Each mouse receives a single daily dose, administered as a bolus into alternate hind limbs for the duration of the study. Hypodermic needles used for im injection should be of the smallest diameter that will

allow injection, but in the range of 27-gauge up to a maximum of about 23-gauge. Prior to dosing, the same procedures for formulation, analysis, weighing of mice, and calculation of doses should be followed as those recommended for ip dosing. The dose is drawn into a syringe, and air bubbles are expelled. The mouse may be held by an assistant, and the needle inserted to the approximate center of the muscle mass. The dose is injected as a bolus, and the needle is withdrawn. The muscle may be massaged gently to distribute the dose prior to returning the mouse to its cage.

Subcutaneous Injection

The sc route of administration is not commonly used in toxicity testing, but may be appropriate if the test article is intended for sc administration to humans, or as a more practical substitute for im testing in mice. The sc route is similar in many characteristics of absorption to the im route and generally results in slower absorption of a test article, with lower peak plasma levels, but more sustained effects than ip administration. The rate of sc absorption can also be influenced by the amount of vascular perfusion of the tissue surrounding the injection, the vehicle, and the injected volume, which indirectly may alter the surface area of tissue available for absorption. Coadministration of a vasodilator generally increases the rate of absorption, whereas coadministration of a vasoconstrictor generally decreases that rate. Administration of the test article as a solution or suspension in a viscous, poorly absorbed vehicle generally retards absorption. The ability to control the rate of absorption is similar to that seen with im injection and can offer the same advantages, as it may allow the toxicologist to administer a dose of a test article that may be absorbed over a period of many hours or even days. Some limitations to im dosing do not apply to sc dosing. sc doses can be injected at a wide variety of sites, if necessary. In addition, dose volumes of up to 10–20 mL/kg/day may be administered repeatedly if they are well absorbed and do not cause excessive local irritation. These large dose volumes allow administration of much higher total doses than can be administered im. It may still be necessary to conduct a separate study to assess sc irritation potential prior to initiation of a repeated-dose toxicity study. Subcutaneous injections can easily be administered to mice without assistance.

Subcutaneous injections are administered into the region beneath the skin using a hypodermic needle attached to a graduated syringe. Each mouse receives a single daily dose, administered as a bolus. Consecutive daily doses may be administered at the same site if absorption is complete and irritation is minimal, but sc trauma may be reduced if the injection site can be changed from day-to-day. Hypodermic needles used for sc injection should be the smallest diameter that will allow injection, but in the range of about 26-gauge up to a maximum of about 20-gauge. While larger volumes can be administered, dose volumes of about 10 mL/kg/day are preferable.

Prior to dosing, the same procedures for formulation, analysis, weighing of mice, and calculation of doses should be followed as those recommended for ip dosing. The dose is drawn into a syringe, and air bubbles are expelled. The mouse is grasped by a fold of skin. One of the most convenient injection sites is the skin in the mid-scapular region, which allows the restraint and dosing of the mouse with minimal risk of being bitten. The needle is inserted through the skin into the sc region. The dose is injected as a bolus, and the needle is withdrawn. The injection site may be pinched for a few seconds to prevent leakage, and the area around the injection site may be massaged gently to distribute the dose prior to returning the mouse to its way.

Intradermal Injection

Intradermal injection is not a route that is commonly used for toxicity studies. It may be appropriate to test products intended for id administration to humans by that same route in mice, and studies of limited duration are technically feasible. The id route offers the advantage of slow absorption owing to the poor vascular perfusion of the skin relative to tissues in other areas of

potential administration. This slow absorption is typically associated with longer time to onset of effects, lower peak plasma levels, but more sustained effects than routes that result in faster absorption. To the extent that the test article may be metabolized by the skin, the id route would be expected to offer greater opportunity for such metabolism than sc injection, but less than topical administration. Injected volume for id dosing should be limited to about 1 mL/kg per injection site or less, with smaller volumes preferred if repeated doses will be administered. It is acceptable to administer id doses at multiple sites simultaneously if higher total doses are required. Irritating formulations of the test article must be avoided, especially if multiple doses will be administered, as ulceration and necrosis of the skin can result.

Intradermal injections can be administered at a variety of accessible sites, but the skin of the abdomen or back is often used. The area in which the injections will be administered should be shaved with a small animal clipper to allow good visualization during and after dosing. Doses can be administered using a small hypodermic needle attached to a graduated tuberculin syringe. Needle diameter should be limited to 27-gauge or smaller, and 30-gauge is preferable. The use of a needle with an id bevel is not necessary. Prior to dosing, the same procedures for test article preparation and analysis of formulations, weighing of mice, and calculation of doses should be followed as recommended for ip dosing. The dose is drawn into the syringe, and air bubbles are expelled. The mouse is held in one hand, and the needle is inserted into the skin at a shallow angle with the bevel of the needle up to avoid penetration into the sc space. With practice, the toxicologist can feel the needle penetrate into the sc space, if that happens by accident, and can relocate the needle prior to injection. A mouse with the needle properly inserted for id injection should show a "bleb" or pump. A properly administered id dose will appear as a small bleb on the surface of the skin. A dose administered into the sc space will not appear as a bleb, as the dose will be distributed over a larger area.

Topical Administration

The topical route of administration is occasionally used for toxicity testing. This choice of route may be appropriate for testing the systemic and local toxic effects of substances intended for human topical administration or which are likely to come into accidental contact with human skin. Data suggest that the mouse is one of the less appropriate species for extrapolation of percutaneous toxicity to the human, as the permeability of mouse skin (as well as rat and rabbit skin) is substantially higher than the permeability of human skin (Maibach and Wester, 1989). Nevertheless, topical application to mice may be appropriate in special cases, such as the conduct of MEST for dermal sensitization potential (Gad et al., 1986).

Historically, the skin was perceived as a barrier to absorption. It is now clear that lipophilic compounds are readily absorbed into and across the skin, and further that the skin may be a source of significant metabolism of some chemicals (Maibach and Wester, 1989). Variables in addition to lipophilicity that are likely to affect dermal absorption include the integrity of the skin at the treatment site; the vehicle employed for dosing, occlusion, and/or restraint of the mouse after treatment; and whether the test article is washed off after some prescribed period. Variations on the integrity of the skin include totally intact skin, skin from which the outer epidermal layers have been tape stripped using a surgical adhesive tape (e.g., Dermiclear), and skin that has been abraded. The presumption is that nonlipophilic test articles will penetrate stripped (thinned) or abraded (interrupted) skin more extensively than they would intact skin. The proper choice of a vehicle may enhance the permeability of the skin to a nonlipophilic chemical. Occlusion of the treatment site and/or restraint of the mouse after application of a topical dose improve retention of the dose in contact with the skin and reduce the probability that the animal will orally ingest the topical dose. Washing excess test article from the treatment site after a prescribed time will limit the exposure period to a known interval. The appropriate choices for these (and other variables) in topical

toxicity study design are a function of the specific objectives of the study, and the physical and chemical characteristics of the test article.

As the number of procedural variables for topical dosing is so great, the procedures described for topical dosing in the MEST (Gad et al., 1986) will be described as a representative technique. In that procedure, the hair is clipped from the treatment site (e.g., abdomen or back) on the first day of treatment, and the epidermal layer is tape stripped until the site has a slightly shimmy appearance, typically 10–20 applications and removals of a surgical adhesive tape such as Dermiclear® (Johnson & Johnson Co., New Brunswick, New Jersey). Next, a fixed volume, e.g., 100 µL, of the test article in a volatile solvent such as ethanol is applied to the treatment site. The solvent is allowed to dry using a warn air blower if necessary, and the animal is returned to its cage. On subsequent treatment days, the tape-stripping operation can be reduced to about 5–410 applications of adhesive tape to achieve the shiny appearance.

Inhalation

The inhalation route of administration offers the most rapid absorption of most test articles, with systemic availability second only to iv injection. Efficiency of absorption by the inhalation route is conferred by the large surface area of the respiratory system, the close proximity of the inner alveolar surface to the blood circulating through the lungs, and the fact that the entire cardiac output passes through the lungs with each circuit of the blood through the body. Absorption of inhaled agents proceeds via one or more of the following mechanisms depending upon specific characteristics of the agent: direct absorption into the bloodstream, absorption from the gastrointestinal tract following deposit in the nasopharynx or transport by mucociliary escalation and swallowing, and/or lymphatic uptake following deposit in the alveoli.

Inhalation studies are particularly useful in estimating the risk of accidental or occupational exposure to a gas, vapor, dust, fume, or mist as well as in evaluating the toxicity of agents that are intended to be administered by inhalation. Administration by inhalation is the most technologically complex means of routine exposure, and a comprehensive description of the procedures is beyond the scope of this chapter. The reader is referred to other works (e.g., Kennedy and Trochimowicz, 1982; McClellan and Henderson, 1989; Menzel and McClellan, 1980) for further description. Rather, this discussion will be limited to some of the advantages, disadvantages, and variables to be considered in inhalation testing. The primary advantage of inhalation is the rapid effective absorption. The primary disadvantage is the technological complexity of the method, with the associated risk of technical error and disregard of an important variable.

For a mouse to inhale a test article, the mouse must be placed in an environment that contains the test article in the form of a gas, vapor, dust, fume, or mist. The test article must exist in a particle size that is inspirable, generally having an aerodynamic diameter from 1 to about 10 A. Particle size dictates where in the respiratory tract the test article will be deposited and absorbed. Larger particles are deposited in the nasopharyngeal region, with successively smaller are particles deposited in the trachea, bronchial, bronchiolar, and finally the alveolar region for particles of about 1 µm or less. The technology of particle generation and uniform distribution throughout the exposure apparatus is complex in itself. In addition to generating and uniformly distributing the test atmosphere, are must be exercised to capture the exhaust from the exposure apparatus, such that the test article can be contained without contamination of the laboratory or environment. Exposure periods can range from a few minutes, appropriate for test articles that may pose only an acute exposure risk, to continuous exposure over a prolonged period, appropriate for test articles that may pose a risk of long-term environmental or occupational exposure. Exposure apparatus generally takes the form of a chamber that contains the whole animal or groups of animals, or a device that exposes only the head or nose of the animal(s) to the experimental atmosphere.

Chamber (Whole Body)

Inhalation chambers allow relatively large numbers of mice to be exposed simultaneously without restraint. The aerodynamic considerations are complex, but simpler than for a head-only or nose-only exposure system. The flow rate through a chamber must be adequate to provide temperature and humidity control. Disadvantages of whole-body chambers include the tendency for test article to accumulate in the fur, from which it can be ingested; on the skin and eyes, which may interfere with the intended route of exposure; and the difficulty in monitoring respiratory volume and rate of individual animals.

Head/Nose Exposure (Head Only/Nose Only)

Head- or nose-only exposure apparatus limits exposure of the mouse to the test article by routes, other than inhalation, as only a small amount of skin and fur are exposed to the test environment. In addition, it is possible to monitor respiratory volume and rate of individual animals with some of the head- or nose-only equipment. Disadvantages to this equipment include the fact that only a relatively small number of animals can be simultaneously exposed, and those animals must be restrained in a position that keeps their heads or noses in close contact with the exposure apparatus. This restraint imposes stress on the animals and virtually precludes continuous exposure, as the processes of eating and drinking are not possible with most of this equipment. The restrainer may limit the animals' ability to dissipate excess body heat.

Data Collection Techniques

Types of data that are routinely collected during the conduct of toxicity studies in mice fall into three broad categories: clinical observations and physical examinations, clinical laboratory evaluations, and postmortem procedures. Cardiovascular parameters are not measured in routine toxicology studies. Heart rates in awake mice have been measured in the range of 300 to more than 800 beats/min (Kaplan et al., 1983). Reliable blood pressure measurements are best made by cannulation of a major artery, such as the carotid. Such procedures require anesthesia and surgery, neither of which is especially desirable during the course of a study that may be of long duration and involve many animals.

Clinical Observations and Physical Examinations

Clinical observations entail the recording of effects that can be detected by direct observation, such as abnormal gait and body weight. For the sake of this discussion, a variety of parameters that can be observed or measured directly will be discussed in this section. Clinical observations often provide the first indication of which physiological systems are being affected by the test article.

Mice should be observed regularly throughout the in-life portion of a toxicity study. The type and frequency of these observations should be tailored to meet the scientific objectives of the specific study. Most effects observed following administration of acute (single) doses occur within a relatively short time after dosing. As acute iv doses are often associated with almost immediate effects, it might be appropriate to observe treated mice within 5 min, at about 15, 30, and 60 min, and again at 2 and 4 hours after dosing. Observations should be repeated at least once daily on all subsequent study days throughout the post-dosing observation period. This schedule should provide information on the times of onset, peak activity, and remission from toxic effects as well as information on the sequence and severity of effects observed. The high intensity of data collection on the day of dosing in acute studies requires that the system for conducting and recording observations

be simple and time efficient. Typically, a system of "exception reporting" is used, in which observations of exceptions from the norm are recorded, and the absence of comment on a system (e.g., respiration) implies that parameter is normal. Clinical observations in repeated-dose studies should be conducted at approximately the same time each day to assure that changes in findings over the course of the study can be attributed to the accumulation of or adaptation to toxic effects rather than incidental changes attributable to circadian rhythm or time after dosing. Minimally, all animals should be observed early in the day, prior to daily dosing, and it is highly desirable to conduct at least one additional daily observation at 2–4 hours after dosing (or late in the day) to be aware of effects that may be associated with higher blood levels of test article usually found from a few minutes to a few hours after dosing.

The simplest form of clinical observation is an observation for survival and moribundity. This or a higher level of observation must be conducted at least once daily in all toxicity studies. The next level of observation is an observation for clinical signs of toxicity, such as abnormal level of spontaneous motor activity, abnormal gait, abnormal respiration, and abnormal quantity or quality of fecal output.

The next level of observation is more structured and is typically conducted about once weekly during studies of a few weeks' duration to as infrequently as about once monthly during the later phases of 26-week to 2-year studies. During the conduct of a physical examination, specific parameters are evaluated, such as quality of coat, body orifices (for excessive or unusual discharges), eyes, and respiratory sounds, and in studies longer than about 26 weeks, animals are examined carefully for evidence of visible or palpable masses. Body weight and feed consumption are typically monitored in studies longer than a few days. An appropriate interval for measuring body weight and feed consumption is about a week. These two parameters should be measured concurrently, such that changes in one can be compared directly to changes in the other. In longer studies, in which the mice have reached maturity and body weight gain has approached zero, the frequency at which body weight and feed consumption are measured can be reduced to as infrequently as once per month. The interval over which they are measured would remain at about a week, however.

Clinical Laboratory Evaluations

Clinical laboratory evaluations of mice refer to evaluations of blood and urine. Blood is routinely collected at sacrifice in repeated-dose studies, and small quantities (e.g., about 0.10 mL) of blood can be collected at interim periods during the course of the study for the purpose of evaluating differential smears or other limited objectives. Interim (nonterminal) blood samples can be collected by retro-orbital venous plexus puncture, cardiac puncture, and tail snip, among other techniques. Each of these techniques has certain disadvantages. Retro-orbital puncture is technically difficult and may require anesthesia or immobilization of the animal. Cardiac puncture typically requires anesthesia, and cardiac injury may compromise the histological evaluation of cardiac tissue. Tail snip often yields samples that are contaminated with extravascular, extracellular fluids. Any administration of anesthetic agents during the study of a test article that is not thoroughly understood engenders some risk to the interpretation of the study, as potential interactions of the anesthetic with the metabolism or direct effect of the test article are nearly impossible to predict. Blood collected at the time of sacrifice is typically drawn from the inferior vena cava or the abdominal aorta while the mouse is under anesthesia. In the case of terminal blood collection, potential interaction of the anesthetic agent with the test article, induction of liver enzymes, etc., is not an issue.

Parameters evaluated in blood samples drawn from mice include evaluation of differential smears for morphological abnormalities and differential white counts, measurement of serum glucose and urea concentrations, serum aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase activity. Red blood cell counts, white blood cell counts, and hemoglobin concentrations can be measured, but these parameters are quite variable for mice on many of the

commonly used laboratory instruments, so they are often omitted. In addition, bone marrow smears may be prepared, but are usually prepared only at sacrifice in mice. Bone marrow smears may help in understanding hematological changes. Caution should be exercised in comparing experimental data with results obtained from the literature or with results obtained on different instrumentation or by different procedures. For greatest utility, a set of normal values should be compiled for the laboratory procedures and equipment used to produce the data in the toxicity study.

As a practical matter, urine is not usually collected in routine toxicity studies. The primary difficulty in conducting urinalysis is that the mouse produces a very small volume of urine during a reasonable collection period (e.g., 16–24 hours), and of that volume, considerable and variable quantities are lost to evaporation and on the surfaces of the collection apparatus. As a result, attempts to evaluate urinary concentrations of practically anything can be very misleading.

Postmortem Procedures

Postmortem procedures, literally those procedures performed after the death of the animal, include confirmation of the identification number and sex of the animal, an external examination, examination of the significant internal organs in place prior to removal, then removal, weighing of appropriate organs, and collection of tissue specimens for histological processing and microscopic examination. The microscopic examination of tissue specimens by a qualified veterinary pathologist may be the single most important source of information in understanding the toxicity of a test article. The pathologist's findings should be carefully integrated with the other study data (e.g., clinical observations, body weights, feed consumption, and clinical laboratory findings) to fully comprehend the effects of the test article on the mouse under the conditions of study.

Ordinarily, the list of tissues to be routinely weighed, collected, and processed for histological examination will be specified in the study protocol. In addition to the tissues specified in the protocol, specimens are usually collected of all lesions or target organs that have been identified during the course of the study or at gross necropsy. It is important to provide the necropsy staff with a current list of abnormal clinical observations, especially any evidence of visible or palpable masses, as this is the time when the visible and palpable lesions can be linked to the histopathological evaluation of those lesions. Every effort should be made to locate all lesions described and collect representative tissue from those sites.

A detailed description of necropsy procedures is beyond the scope of this discussion. It should be emphasized that the necropsy process, particularly when conducted on a large number of animals at the scheduled termination of a study, is a process in which a large number of tissue samples may be collected, and a similarly large quantity of data may be gathered during a short period of time. As such, this process presents many opportunities for loss or misidentification of samples and data. A rigorous system of accounting for which tissues have been collected from each animal, and for tracking the samples and data collected, is critical to the accurate interpretation of the toxicity study.

Summary

In summary, the mouse is one of the most useful species for toxicity testing. Mice have been used in biomedical research for hundreds of years. As a result of long usage, many techniques have been developed to dose and evaluate mice, and a wealth of historical data has been accumulated in the literature. A wide variety of genetic strains have been developed for specific purposes. It is often possible to select a strain for testing that is particularly vulnerable or resistant to either the test article or a particular type of lesion that might be expected to be associated with that test article. The small size of the mouse confers economy in acquisition, husbandry, handling, and test article consumption. The relatively short gestation period and life span of the mouse are useful in conducting

reproductive studies or studies in which the test article will be administered for a high percentage of the lifetime of the animal.

The small size of the mouse is responsible for most of the disadvantages of the species as well. The species is relatively susceptible to environmental stress. Small size and blood volume make it difficult or impossible to collect multiple samples of blood and urine over short periods of time. Assays that might require large volumes of blood or urine are precluded. Certain physiological evaluations, such as electrocardiograms, are difficult owing to the small size and high activity level of the species.

PATHOLOGY

Charlie Clifford and Dawn Goodman

Introduction

As they age, animals of all species, including humans, develop a variety of lesions, both neoplastic and nonneoplastic. Such lesions are usually referred to as spontaneous or age-associated lesions. These lesions are most frequently proliferative, degenerative, or inflammatory in nature. Each species and strain of animal has certain characteristic lesions, which are commonly observed with aging. The purpose of this chapter is to discuss those age-associated lesions frequently observed in various strains and stocks of mice and those that might interfere with the interpretation of toxicology studies. The B6C3F1 hybrid mouse strain and the CD-1 mouse stock are the two strains/stocks of mice most commonly used in toxicology studies in the United States. The National Toxicology Program (NTP) utilizes the B6C3F1 mouse, whereas private industry utilizes primarily the CD-1 strain for toxicology studies. Both strains have their advantages and their disadvantages (Maronpot, 1999). Data are available regarding the time of onset and incidences of age-associated lesions found in these strains/stocks. This chapter will primarily discuss lesions found in B6C3F1 and CD-1 mice. There is a section on transgenic animals that are becoming increasingly important in toxicology studies.

The B6C3F1 mouse is the F_1, hybrid of two inbred strains, C57BL/6 females and C3H/HeN males, and is designated (C57BL/6 × C3H/HeN)F_1. The C57BL/6 strain was originated by Little in 1921. The C3H strain was originated by Strong in 1920 (Anonymous, 1981). When the C3H strain was obtained by Heston in 1941, it was designated as the C3H/HeN. Subsequently, the strain was established behind the SPF barrier. The mouse mammary tumor virus (MTV), which is transmitted through the milk, was not transmitted to the offspring, which were caesarean derived and maintained behind the barrier. The strain was then designated as C3H/HeN-MTV-. This strain is used to provide the male parent for the B6C3F1 mouse used by the NTP and, therefore, does not carry the MTV (Goodman et al., 1985). The B6C3F1 mouse from the Charles River Laboratories is likewise MTV negative (Anonymous, 1982). However, it is possible that B6C3F1 mice from other sources may be derived from C3H mice carrying the MTV.

The CD-1 mouse is an outbred stock maintained at the Charles River Laboratories. The full designation for this stock is Crl: CD-1 (ICR) BR. The CD-1 mouse was derived from Swiss stock (Anonymous, 1982). The so-called Swiss mouse was imported to the United States in 1926 by C. J. Lynch of the Rockefeller Institute for Medical Research and was derived from a noninbred stock maintained in Europe. Subsequently, the stock was given to other researchers and commercial laboratories (Lynch, 1969). The Swiss stock used by the Charles River Laboratories to establish the CD-1 mouse was obtained from Hauschka and Mirand of Roswell Park Memorial Institute (HaM/ICR) in 1959. The CD-1 mouse was caesarean derived at that time and, subsequently, maintained behind an SPF barrier (Anonymous, 1982). As we have come to better know the genome of the mouse (mouse genomic information—http://www.informatics.jax.org), our understanding of the applicability of different models has expanded (Conn, 2013).

In evaluating toxicology studies, it is important to be aware of the types of age-associated lesions observed in untreated animals of the strain/stock of mouse used in the study, at what age such lesions are generally observed, the incidences observed, and the degree of severity (Haschek et al., 2013). Generally, few, if any, lesions are encountered in CD-1 and B6C3F1 mice less than 6 months of age. Those that are encountered are usually sporadic in occurrence and minimal to mild in severity. Neoplasms of any type are rarely seen in animals of this age, although they can occur. Most acute and subchronic toxicity studies in mice are conducted in animals less than 6 months of age. Thus, spontaneous lesions rarely complicate interpretation of data from these types of studies. After a year of age, spontaneous lesions start to appear, increasing in frequency and severity with advancing age. Most long-term toxicology studies are terminated at 18 months or 2 years with CD-1 or B6C3F1 mice, respectively. B6C3F1 mice have good longevity with 75%–80% surviving to the end of 2-year studies (Goodman et al., 1985). At 2 years, only 50% of CD-1 mice are still alive (Anonymous, 1982).

For the B6C3F1 mouse, extensive data have been published on the incidences of spontaneous neoplasms (Anonymous, 1989; Goodman et al., 1985; Haseman et al., 1984, 1985, 1998; Ward et al., 1979a).

When using databases such as the one compiled by the NTP, it is important to apply the correct scientific principles in the evaluation of these data (Haseman, 2000). Although the CD-1 mouse is used in many laboratories that have developed their own databases for disease incidences, relatively little data have been published in a concise form (Homburger et al., 1975; Lang, 1987; Percy and Jonas, 1971). For both the CD-1 and the B6C3F1 mice, there are little published data on the incidences of nonneoplastic diseases.

It is well known that factors such as nutrition, husbandry (for example, individual vs. group housing), and genetic background can affect the development of many spontaneous lesions and alter the tumor profile (Haseman et al., 1994). Necropsy protocols, tissue sampling procedures, and diagnostic criteria can also have an impact on the reported incidences of lesions; thus, it is important that each laboratory develops its own historical incidence data. Primary emphasis should be given to concurrent rather than historical control data when interpreting experimental results (Haseman et al., 1997). Nomenclature and diagnostic criteria have been published for neoplastic lesions, providing some degree of standardization. For nonneoplastic lesions, nomenclature and diagnostic criteria are not well established. In addition, some pathologists may use a single diagnosis for a specific disease entity, e.g., nephropathy, whereas others may diagnose each of the components of the disease process. Some pathologists "read through" common spontaneous (background) lesions, particularly if the lesions are minimal to mild in severity. All of these factors make it difficult to compare incidences of spontaneous nonneoplastic lesions between laboratories and even between studies evaluated by different pathologists.

Since there is some standardization of neoplastic lesions, incidence data derived from other laboratories for specific neoplasms for untreated animals of the same strain and sex can be relevant. Data from different laboratories can be used to determine whether the in-house laboratory controls really do reflect the incidences of specific neoplasms in the overall population. Such controls are also useful when little in-house laboratory data are available. With this in mind, historical control data for neoplasms with an incidence of greater than 0.5% are presented for CD-1 mice (Table 2.22). Historical control data for B6C3F1 mice include tumors of incidences as low as 0.1%.

The historical control data presented in Tables 2.20 and 2.21 for B6C3Fl mice are based on data from the NTP (Anonymous, 2000; Haseman et al., 1999). These data are derived from studies conducted at several contract laboratories. Table 2.20 reflects data from animals fed the NTP 2000 diet, whereas Table 2.21 reflects data from animals on NIH07 diet. Although the data are collected from multiple laboratories, some variables have been minimized. The derivation and source of the animals as well as the diet used are controlled by the NTP. The data include only untreated controls. The data are based on all animals placed on study regardless of date of death.

Table 2.20   Incidences of Tumors in Untreated Control B6C3F1 Mice Fed NTP 2000 Diet in 2-Year Studies

NTP Diet 2000	Male		Female	
	Number of Tumors (%)	Range (%)	Number of Tumors (%)	Range (%)
Circulatory system	659[a]		659[a]	
Hemangioma	5 (0.8)	0–4	8	0–5
Hemangiosarcoma	37 (5.6)	2–14	15	0–8
Digestive tract				
Liver	659[a]		655[a]	
Hepatocellular adenoma	195 (29.6)	12–46	101 (15.4)	8–28
Hepatocellular carcinoma	145 (22)	13–46	49 (7.5)	3–16
Forestomach	659[a]		659	
Squamous cell papilloma	11 (1.7)	0–6	10 (1.5)	0–6
Small intestine	659		659	
Adenoma	3 (0.5)	0–4	1 (0.2)	0–2
Carcinoma	15 (2.3)	0–10	1 (0.2)	0–2
Endocrine system				
Adrenal cortex	655[a]		649[a]	
Cortical adenoma	24 (3.7)	0–10	1 (0.2)	0–2
Adrenal medulla	653[a]		644[a]	
Pheochromocytoma benign, complex, malignant, NOS	3 (0.5)	0–2	6 (0.9)	0–2
Pituitary site unspecified	622[a]		623[a]	
Adenoma	3 (0.5)	0–2	59 (9.5)	0–20
Carcinoma	1 (0.2)	0–2	0	0
Thyroid	651[a]		646[a]	
Adenoma	1 (0.2)	0–1	2 (0.3)	0–2
Carcinoma	1 (0.2)	0–2	0	0
Hematopoietic system	659[a]		659[a]	
Lymphoma/leukemia	33 (5)	2–8	98 (14.9)	6–32
Integumentary system	659		659	
Epithelial neoplasms	2 (0.3)	0–2	1 (0.2)	0–1
Fibroma, sarcoma, or fibrous histiocytoma	1 (0.2)	0–1	21 (3.2)	0–6
Musculoskeletal system	659[a]		659[a]	
Rhabdomyosarcoma	0	0	3 (0.5)	0–3
Osteosarcoma	0	0	2 (0.3)	0–2
Reproductive system				
Ovary			626[a]	
Cystadenoma			24 (3.8)	0–16
Granulose cell tumor			5 (0.8)	0–3
Luteoma			4 (0.6)	0–3
Uterus			659[a]	
Endometrial stromal polyp			11 (1.7)	0–6
Mammary gland	659[a]		659[a]	
Fibroma, fibroadenoma, carcinoma, or adenoma	0	0	2 (0.3)	0–2
Respiratory system	659[a]		654[a]	
Alveolar/bronchiolar adenoma	119 (18.1)	4–26	37 (5.7)	0–12
Alveolar/bronchiolar carcinoma	68 (10.3)	8–24	17 (2.6)	0–6
Harderian glands	659[a]		659[a]	
Adenoma	53 (8)	2–16	41 (6.2)	0–20
Carcinoma	4 (0.6)	0–4	9 (1.4)	0–4

[a] Number of tissues examined.

Table 2.21 Incidences of Tumors in Untreated Control B6C3F1 Mice Fed NIH07 Diet in 2-Year Studies

NIH07 Diet	Male Number of Tumors (%)	Range (%)	Female Number of Tumors (%)	Range (%)
Circulatory system	1355[a]		1353[a]	
Hemangioma	7 (0.5)	0–4	22 (1.6)	0–8
Hemangiosarcoma	73 (5.4)	0–12	37 (2.7)	0–8
Digestive tract				
Liver	1350[a]		1350[a]	
Hepatocellular adenoma	397 (29.4)	4–60	234 (17.3)	2–50
Hepatocellular carcinoma	241 (17.9)	6–29	113 (8.4)	0–20
Forestomach	1355[a]		1353[a]	
Squamous cell papilloma	18 (1.3)	0–6	20 (1.5)	0–6
Small intestine	1355[a]		1353[a]	
Adenoma	5 (0.4)	0–4	2 (0.1)	0–2
Carcinoma	12 (0.9)	0–6	8 (0.6)	0–6
Endocrine system				
Adrenal cortex	1335[a]		1347[a]	
Cortical adenoma	46 (3.4)	0–22	9 (0.7)	0–6
Adrenal medulla	1330[a]		1330[a]	
Pheochromocytoma	11 (0.8)	0–5	14 (1.1)	0–4
Pituitary (pars distalis)	1265[a]		1290[a]	
Adenoma	5 (0.4)	0–6	185 (14.3)	0–36
Carcinoma	0	0	6 (0.5)	0–4
Thyroid	1343[a]		1340[a]	
Follicular cell adenoma	20 (1.5)	0–4	24 (1.8)	0–8
Follicular cell carcinoma	6 (0.4)	0–2	2 (0.1)	0–2
Hematopoietic system	1355[a]		1353[a]	
Lymphoma/leukemia	113 (8.3)	2–20	284 (21)	0–42
Integumentary system	1355[a]		1353[a]	
Epithelial neoplasms	6 (0.5)	0–2	2 (0.2)	0–2
Fibroma, fibrosarcoma, sarcoma, myxoma, myxosarcoma, or fibrous histiocytoma	83 (6.1)	0–24	33 (2.4)	0–8
Musculoskeletal system	1355[a]		1353[a]	
Osteosarcoma	1 (0.1)	0–2	8 (0.6)	0–4
Reproductive system				
Ovary			1323[a]	
Adenoma			7 (0.5)	0–4
Cystadenoma			19 (1.4)	0–4
Granulose cell tumor			10 (0.8)	0–4
Luteoma			3 (0.2)	0–2
Teratoma			6 (0.5)	0–2
Uterus			1353[a]	
Endometrial stromal polyp			44 (3.3)	0–16
Mammary gland	1355[a]		1353[a]	
Carcinoma or adenoma	0	0	9 (0.6)	0–2
Respiratory system	1354[a]		1352[a]	
Alveolar bronchiolar adenoma	217 (16)	4–30	80 (5.9)	0–24
Alveolar bronchiolar carcinoma	69 (5.1)	0–14	32 (2.4)	0–8
Harderian glands	1355[a]		1353[a]	
Adenoma	64 (4.7)	0–18	45 (3.3)	0–10
Carcinoma	9 (0.7)	0–4	9 (0.7)	0.4

[a] Number of tissues examined.

The NTP conducts an extensive review of the pathology portion of their studies for neoplastic lesions. Thus, the terminology for neoplastic lesions is generally comparable between studies and laboratories (Goodman et al., 1985).

The historical control data presented in Table 2.22 for CD-1 mice have been obtained from Hazleton Washington, Vienna, Virginia (courtesy of S. Jones and S. Weymouth). All studies were conducted between 1984 and 1989 and were dietary studies lasting approximately 78 weeks. The data are based only on animals killed at terminal sacrifice and do not include animals dying on study.

As mentioned earlier, there are little published data on the incidences of spontaneous nonneoplastic lesions in mice. Some of the more common lesions observed in CD-1 and B6C3F1 mice as well as some of the more common lesions in other strains of mice are described in the text, although incidences are generally not given. With current husbandry practices, infectious diseases are uncommon in laboratory mice. Important infectious diseases are briefly described where applicable.

At necropsy, organ weights are usually taken for a number of organs. Historical control data on this information are sadly lacking in the literature. These data are included for B6C3F1 and CD-1 mice in this chapter. There can be problems in interpreting such data, and these are discussed in the section on organ weights.

Cardiovascular System

Vessels

Nonneoplastic Lesions

Vascular Ectasia (Angiectasis) — Vascular ectasia can occur in a variety of organs and consists of dilatation of the capillaries in a focal area. Microscopically, angiectasis consists of dilated vascular spaces or sinusoids lined by flattened or slightly plump endothelial cells. This lesion is frequently seen in the ovary of aged mice. Vascular ectasia is also often seen in the mesenteric lymph node. The affected lymph nodes are usually enlarged and dark red. Microscopically, there are dilated vascular channels filled with red blood cells and lined by well-differentiated endothelium. The lining of the vascular channels is sometimes incomplete. The lesion is more prominent in the medulla but can affect the cortex and, occasionally, the perimesenteric fat as well. This lesion has been referred to as mesenteric disease (Dunn, 1954). Vascular ectasia, in any organ, must be distinguished from vascular tumors (i.e., hemangioma or hemangiosarcoma). The absence of endothelial cell proliferation and/or nuclear atypia differentiates angiectasis from neoplastic vascular lesions (hemangioma and hemangiosarcoma).

Polyarteritis — Polyarteritis in the mouse has been compared to periarteritis nodosa in man. The etiology of both is unknown, but an immune origin is suspected. In mice, the lesion involves small muscular arteries and is usually evident in multiple sites. Organs commonly involved include the heart, tongue, uterus, testis, kidney, and urinary bladder. The media of the affected vessels is homogeneous and intensely eosinophilic with hematoxylin and eosin (H&E) stain. Both fibrosis and an infiltration of mononuclear cells occur around the affected vessels. This disease is uncommon in the B6C3F1 and CD-1 strains, but it has been reported in BALB/c mice (Frith and Ward, 1988).

Neoplastic Lesions

General — Tumors of endothelial cells (hemangioma, hemangiosarcoma) can be found at any site in the body. The most common sites are the spleen and liver. The subcutis, skeletal muscle, and female reproductive tract are also other common sites (Strandberg and Goodman, 1982). Lymphangiomas and lymphangiosarcomas, tumors of lymphatics, are rare.

Table 2.22   Incidences of Primary Tumors in Untreated Control CD-Mice[a] 18-Month Studies[b,c]

	Male		Female	
	Number of Tumors (%)	Range (%)	Number of Tumors (%)	Range (%)
Circulatory system	(363)[d]		(391)[d]	
Hemangioma	5 (1.4)	0–8	5 (1.3)	0–3
Hemangiosarcoma	9 (2.5)	0–6	10 (2.6)	0–12
Digestive tract				
Liver	(362)[d]		(391)[d]	
Hepatocellular adenoma	35 (9.7)	0–16	5 (1.3)	0–5
Hepatocellular carcinoma	19 (5.2)	0–16	2 (0.5)	0–3
Endocrine system				
Adrenal	(362)[d]		(391)[d]	
Cortical adenoma	3 (0.8)	0–5	0 (0.0)	—
Pituitary	(362)[d]		(391)[d]	
Adenoma	0 (0.0)	—	3 (0.8)	0–3
Thyroid	(362)[d]		(391)[d]	
Follicular cell adenoma	2 (0.6)	0–4	0 (0.0)	—
Follicular cell carcinoma				
Hematopoietic system	(362)[d]		(391)[d]	
Mixed-cell lymphoma	2 (0.6)	0–5	6 (1.5)	0.6
Histiocytic sarcoma (lymphoma)	0 (0.0)	—	4 (1.0)	0–2
Lymphocytic lymphoma	8 (2.2)	0–11	15 (3.8)	0–17
Reproductive system				
Testis	(362)[d]			
Interstitial cell tumor	3 (0.8)	0–5		
Ovary			(391)[d]	
Cystadenoma			3 (0.8)	0–5
Luteoma			4 (1.0)	0–3
Uterus			(391)[d]	
Endometrial stromal polyp			14 (3.6)	0–10
Endometrial stromal sarcoma			2 (0.5)	0–3
Leiomyoma			7 (1.8)	0–4
Leiomyosarcoma			5 (1.5)	0–5
Mammary gland	(362)[d]		(391)[d]	
Fibroadenoma	0 (0.0)	—	1 (0.3)	0–4
Adenocarcinoma	0 (0.0)	—	4 (1.0)	0–3
Respiratory system	(362)[d]		(391)[d]	
Alveolar bronchiolar adenoma	47 (13.0)	0–30	33 (8.4)	0–19
Alveolar bronchiolar carcinoma	10 (2.8)	0–8	8 (2.0)	0–8
Special sense organs				
Harderian glands	(335)[d]		(359)[d]	
Adenoma	7 (2.1)	0–6	3 (0.8)	0–5
Carcinoma	0 (0.0)	—	1 (0.3)	0–2

[a] Tumors with an incidence of 0.5% or greater in one or both sexes.
[b] Includes only terminal sacrifice animals.
[c] Data supplied by Ms. S. Weymouth and Dr. S. Jones of Hazelton Washington, Vienna, Virginia, 1990.
[d] Number of animals examined.

Hemangioma — Hemangiomas are benign tumors arising from endothelial cells. Microscopically, they consist of dilated vascular spaces or thin-walled capillaries lined by a single layer of endothelial cells. The endothelial cells are well differentiated and usually slightly plump. Mitotic figures are scant. The connective tissue stroma is often delicate but may consist of dense collagenous tissue, particularly around the dilated spaces.

Hemangiosarcoma — Hemangiosarcomas are malignant neoplasms arising from endothelial cells. These neoplasms may be pleomorphic. They consist of vascular spaces of varying sizes, which may or may not be filled with red blood cells. The cells lining the vascular spaces are plump with oval basophilic nuclei and with indistinct cell borders. There is often piling up of the lining cells. In areas of the tumor, there may be solid sheets of cells. Particularly in the spleen, the tumors may be predominantly solid. Necrosis, hemorrhage, and thrombosis are frequent. Hemangiosarcomas may metastasize, although it is usually difficult to determine the primary site. Often, there may be multiple primary sites. This is one of the reasons that the incidence of vascular tumors is calculated using the number of animals with vascular tumors regardless of site.

Heart

Nonneoplastic

General — Spontaneous nonneoplastic lesions of the heart are relatively uncommon in CD-1 and B6C3F1 mice.

Cardiomyopathy — This is a spontaneous age-related degeneration frequently evident in the myocardium (Elwell and Mahler, 1999). Early in this process, there is myocyte degeneration or necrosis. The inflammatory component of this lesion varies, and this is not solely a consequence of the age of the lesion. There is frequently an increase in the interstitial fibrous connective tissue of the myocardium. Cardiomyopathy is most common in the ventricles. Cardiomyopathy can be induced by chemicals such as methyl bromide and diethanolamine (Melnick et al., 1994).

Epicardial and Myocardial Mineralization — Mineralization of the myocardium and epicardium occurs in some strains of mice as early as 1 month of age and is more common in males than in females; it occurs most often on the right ventricle. Microscopically, the mineralized foci are characterized by distinctly basophilic areas after H&E staining, by black areas when stained with von Kossa, and red areas when stained with Alizarin Red. The mineralization may be minimal or quite extensive, covering most of the epicardial surface of the right ventricle. The mineralized areas may be surrounded by fibrosis, but an inflammatory component is minimal. The exact etiology is unknown, but the lesion appears to have a genetic predisposition (Brownstein, 1983; DiPaola et al., 1964). Mineralization of the myocardium is also a common lesion in a number of strains of mice (Rings and Wagner, 1972). The mineralized areas are focal and may involve the myocardium of both ventricles and the interventricular septum. The atria are usually spared. The lesion appears to be a distinct entity from the epicardial mineralization. Microscopically, the mineralized areas stain distinctly basophilic with H&E. The focal areas of mineralization are often surrounded by fibrous connective tissue, and a minimal infiltrate of mononuclear inflammatory cells is present.

Atrial Thrombosis — Atrial thrombosis is not a common lesion, but occurs as both a spontaneous and an induced lesion in mice (Schieferstein et al., 1985). The thrombus more commonly involves the left atrium. Grossly, the involved atrium is enlarged and red. Microscopically, the distended atrium contains an organizing mural thrombus. The degree of organization depends upon the age of the thrombus. Some of the thrombi may contain focal areas of cartilaginous metaplasia. If the thrombi are large, they may lead to a secondary chronic passive congestion of the lungs.

Dietary factors are known to influence atrial thrombosis. A high-fat and low-protein diet and copper deficiency have increased the incidence of atrial thrombosis. There is a high incidence of atrial thrombosis in the BALB/c strain of mice. Chemicals, for example, doxorubicin, that induce myocardial damage may also induce atrial thrombosis (Fujihira et al., 1993).

Proliferative Lesions

Hyperplastic Lesions Heart — Proliferation of the capillary endothelium has occurred in females in association with mammary gland adenocarcinomas (Iwata et al., 1994). This endothelial proliferation also occurs as a precursor to chemically induced hemangiosarcomas that are induced by 1,3 butadiene (Melnick et al., 1990).

Neoplasia Heart — Primary tumors of the heart are extremely rare in mice (Strandberg and Goodman, 1982).

Neoplasms that have been found in association with ionizing irradiation are mesenchymoma and mesenchymal cell sarcoma (Hoch-Ligeti and Stewart, 1984). Systemic, metastatic, or locally invasive neoplasms that may be found in the heart are lymphoma, histiocytic sarcoma, and pulmonary alveolar/bronchiolar carcinomas.

Digestive System

Salivary Glands

General

The mouse as well as many other species has three paired salivary glands—submaxillary (submandibular), parotid, and sublingual (Frith and Townsend, 1985). All three major salivary glands are closely associated and located in the sc tissue of the ventral neck area.

Sexual Dimorphism

The submandibular, or submaxillary, salivary gland is distinctly different morphologically in adult male and female mice. Immature mice of both sexes present a pattern similar to that in adult female mice, suggesting that the difference in adult males is due to the production of testosterone. Castration of males before puberty results in loss of the male characteristics of the gland (Botts et al., 1999). In the female mouse, the acini are small, and epithelial cells have centrally located nuclei and only a few cytoplasmic granules. In the adult male, the acini are much larger, and the tall columnar lining epithelial cells have basally located nuclei and abundant eosinophilic cytoplasmic granules. This sexual dimorphism is not present in the rat.

Nonneoplastic Lesions

Lobular Hyperplasia and Atrophy — Lobular hyperplasia and atrophy are seen in both the submaxillary and parotid salivary glands, but are rare in the sublingual. The lesion commonly involves a single lobule in which some acini are atrophied and replaced by hyperplastic ducts.

Basophilic Foci — Chiu and Chen (1986) described the occurrence of basophilic hypertrophic foci in the parotid glands in both Sprague Dawley rats and Swiss mice. They were characterized by focal hypertrophy and hyperchromasia of acinar cells. The incidence in the Swiss mice was 4.8%. The lesions were considered to be spontaneous, and their morphology and growth pattern suggested

that they were neither degenerative, necrotic, hyperplastic, preneoplastic, nor neoplastic. The investigators considered them to be a distinct pathological entity of an unknown nature.

Salivary Mucocele, Duct Dilatation, or Cyst — These may be seen occasionally and are likely to be the consequence of duct obstruction by calculi or foreign bodies.

Inflammatory cell infiltration occurs frequently in B6C3F1 mice. This consists typically of lymphocytes with occasional plasma cells. Suppurative inflammation occurs infrequently.

Neoplastic Lesions

Spontaneous neoplasms of the major salivary glands of mice occur relatively infrequently (Frith and Townsend, 1985). Because mammary tumors often arise in the neck area immediately adjacent to the salivary glands, they have sometimes been mistakenly identified as salivary gland tumors. Careful dissection of a tumor and the contiguous normal tissues in the neck and head region, followed by histological demonstration of actual involvement of a salivary gland by the tumor is, therefore, essential if one is to have reasonably sound evidence of salivary gland origin. Any spontaneous tumor in the neck region of a female mouse of strain BALB/c, A, C58, CF-1, or any hybrid of the C and A strains is most likely to be a salivary gland tumor of myoepithelial type.

Spontaneous neoplasms of the salivary gland are extremely rare in all strains of mice. The NTP Carcinogenesis Bioassay Data System reported only 30 epithelial tumors from 40 chronic studies using the B6C3F1 mouse, representing approximately 8000 animals.

Myoepithelioma

Myoepithelioma of the salivary glands is rare in mice except in certain strains (Delaney, 1977; Peters et al., 1972). It is extremely rare in the B6C3F1 and the CD-1 strains. The neoplasm may occur at a low incidence in the BALB/c mouse and is more common in the female. It develops most frequently in the submaxillary or parotid salivary glands and is rare in the sublingual gland. Myoepitheliomas have also been reported in the human parotid gland (Leifer et al., 1974). The myoepithelioma is believed to be derived from the myoepithelial cells in the salivary glands. The tumors become quite large and grossly are often cystic. Microscopically, they are composed of large pleomorphic cells, suggesting both an epithelial and a mesenchymal origin. The larger tumors commonly metastasize to the lung.

Other Tumors

Spontaneous tumors of the mouse salivary glands other than myoepitheliomas are extremely rare. Mouse polyoma virus causes salivary gland tumors, including a mesenchymal, an epithelial, and a mixed epithelial/mesenchymal type (Dawe, 1979). Polyoma virus infection does not occur as a spontaneous disease.

Adenomas may arise from the serous or mucous acinar cells. The absence of ducts is a feature that distinguishes adenomas from hyperplasia (Betton et al., 2001). Adenocarcinomas may have solid, papillary, or mixed pattern of growth with local invasion and possibly metastases in the lung.

Pancreas

Nonneoplastic Lesions

Acinar Atrophy and Fatty Replacement — Atrophy of the acinar pancreas with fatty replacement occurs at a low incidence in mice and may be lobular or diffuse. The existing islets appear

normal and are embedded in a stroma of adipose tissue. Occasionally, the adipose tissue contains a chronic inflammatory cell infiltrate. The occurrence of the lesion only in aged mice suggests that it is a true atrophy and not hypoplasia. Atrophy occurs at a rate of 1%–2% in 2-year studies in B6C3F1 mice (Boorman and Sills, 1999).

Cysts — These occur rarely and are usually solitary. They are lined by cuboidal to flattened epithelium.

Foci of cellular alteration are found occasionally in mouse 2-year studies. They are composed of adjacent acini that vary in staining intensity from the surrounding parenchyma.

Neoplastic Lesions

Acinar Cell Tumors — Acinar cell tumors have been described in the rat (Boorman and Eustis, 1985), but they are extremely rare in the mouse (Cavaliere et al., 1981; Prejean et al., 1973).

"Acinar cell hyperplasia" is differentiated from acinar cell adenoma by the lack of compression of the adjacent tissue (Deschl et al., 2001).

"Acinar cell carcinoma" is differentiated from acinar cell adenoma by cellular pleomorphism and anaplasia and local invasion or distant meatastases.

Esophagus

Nonneoplastic

Esophageal Rupture — Rupture of the esophagus is seen in mice as a result of faulty oral intubation and gavage. If the animal survives, an associated inflammatory reaction is present. The lesion is usually fatal.

Neoplastic Lesions

Papilloma and Squamous Cell Carcinoma — Papillomas and squamous cell carcinomas have been reported in the rat (Cardesa and Ovelar, 1985; Ovelar and Cardesa, 1985), but appear to be rare in the mouse. The experimental production of papillomas has been reported in the mouse (Horie et al., 1965).

Stomach

Normal Anatomy

The stomach of both the mouse and the rat is divided into a glandular stomach and a nonglandular forestomach. The two regions are separated by a ridge around the entrance of the esophagus. The ridge is formed by the thickened lamina propria of the nonglandular stomach. The nonglandular stomach is lined by stratified squamous epithelium, and the glandular stomach is lined by glandular epithelium. The glandular region contains three types of glands: cardiac, pyloric, and fundic.

Nonneoplastic Lesions

Glandular Hyperplasia — Hyperplasia of the glandular gastric mucosa may occasionally be seen in mice. The lesion may be either focal or diffuse in nature.

Squamous Cell Hyperplasia of the Forestomach — The forestomach (nonglandular stomach) of the mouse is lined by stratified squamous epithelium that may occasionally become hyperplastic.

This lesion may result from the oral administration of toxic irritants. Erosions and ulcers of the forestomach can occur in association with treatment and are more common in mice treated by gavage, by inhalation, or with irritating agents via dosed food and water than in control groups (Leininger et al., 1999).

Cysts — These occur in the esophagus and forestomach and consist of squamous epithelial lining and multiple layers of keratin filling the lumen. Cystic gastric glands, with calcium deposits and inflammation, are a frequent finding in mice in the cardia.

Neoplastic Lesions

Adenoma and Adenocarcinoma of the Glandular Stomach — Adenoma and adenocarcinoma of the gastric mucosa are rare in mice. Adenomas are usually well circumscribed and delineated from the normal mucosa. The cells within the adenomas are well differentiated. Gastric adenocarcinomas are invasive and infiltrate into the lamina propria and muscularis. Neuroendocrine neoplasms (carcinoids or enterochromaffin cell like tumor) may be induced in the mouse and also have occurred spontaneously in B6C3F1 mice in NTP studies.

Squamous Cell Papilloma and Carcinoma of the Forestomach — Hyperplasia and neoplasia of the squamous epithelium are part of a continuum that ranges from focal hyperplasia to papilloma to localized squamous cell carcinoma to invasive squamous cell carcinoma.

Papillomas are composed of a stalk with a vascular connective tissue core covered by neoplastic squamous epithelium. Squamous cell carcinomas are usually relatively well differentiated and produce keratin. They are locally invasive and may occasionally metastasize to the lungs.

Intestine

Nonneoplastic Lesions

Epidermal Inclusion Cysts — Epidermal inclusion cysts are an infrequent finding in the B6C3F1 mouse and are present in the muscularis of the colon and rectum (Shackelford and Elwell, 1999).

"Diverticuli" of the cecum and colon are infrequently detected and must be differentiated from adenocarcinomas.

"Fat necrosis" of mesentery is thought to be associated with torsion.

Intussusception and Rectal Prolapse — Intussusception may occur in both the large and small intestines and may lead to intestinal obstruction, inflammation, necrosis, and death. Prolapse of the rectum may be associated with a pinworm infection or Citrobacter infection (Barthold et al., 1978); it is characterized by an eversion of the mucosal surface of the rectum through the anus.

Pinworms — Pinworms in mice commonly involve two species, *Syphacia obvelata* and *Aspiculuris tetraptera*. They are usually found in the colon, and sections of the parasite may be seen microscopically in the colonic lumen of infected mice. Pinworms are not usually associated with any pathological changes in the large intestine, but occasionally, intussusception or rectal prolapse may occur.

Inflammatory Lesions — The current high standard of husbandry practices for laboratory mice means that infectious diseases are extremely rarely encountered in toxicology studies. *Citrobacter freundii* causes a disease entity known as colonic hyperplasia or transmissible murine colonic hyperplasia. Although this infection is rare, it may complicate carcinogenicity studies as the transitory hyperplasia of the colonic mucous membrane has been found to increase the incidence of tumors

and decrease the dose of carcinogen required for tumor induction. Salmonella infection of the small and large intestine is characterized by multifocal necrosis and thrombosis. Tyzzers disease (*Bacillus piliformis*) produces necrotizing inflammatory lesions especially in the terminal ileum and cecum.

Several viruses cause intestinal lesions in mice: mouse hepatitis virus, EDIM (a group A rotavirus), Reovirus, and mouse adenovirus infections.

Neoplastic Lesions

Adenoma and Adenocarcinoma — Adenomas of the small intestine are frequently small and may not be detected if the intestine is unopened during gross examination. They are especially common in the duodenum.

Microscopically, the adenoma appears as a polypoid epithelial growth projecting into the lumen of the intestine. The epithelium is relatively well differentiated, but usually appears more basophilic than adjacent normal epithelium. Benign neoplasms may develop dysplastic foci and transform into malignant tumors.

Adenocarcinomas are composed of more anaplastic or pleomorphic cells, which may project into the lumen as well as infiltrate into and beyond the submucosa and tunica muscularis. Adenocarcinomas frequently are cystic or papillary and microscopically show many mitotic figures.

Leiomyoma and Leiomyosarcoma — Smooth muscle tumors of the small intestine are relatively uncommon. Leiomyomas are usually well-circumscribed lesions composed of well-differentiated smooth muscle cells. Leiomyosarcomas are not well circumscribed, and neoplastic smooth muscle cells may infiltrate the submucosa and serosa.

Other neoplasms that occur in the intestines are schwannoma, fibroma, fibrosarcoma, hemangioma, hemangiosarcoma, and lymphomas.

Endocrine System

Adrenal Gland

Nonneoplastic Lesions

Subcapsular Cell Hyperplasia — Many strains of mice develop proliferations of spindle cells in the subcapsular region of the adrenal cortex (Dunn, 1970; Goodman, 1983). The cell of origin is the glomerulosa cortical cell.

The proliferating cells may extend downward into and through the zona glomerulosa and zona fasciculata. The cells are fusiform or oval with spindle-shaped nuclei and scant basophilic cytoplasm. The spindle cells have been referred to as type A cells (Dunn, 1970; Goodman, 1983) and are the most common type seen. Type B cells are occasionally found. These are larger polygonal cells with abundant eosinophilic cytoplasm, which is usually lipid-laden and round vesicular nuclei. Large hyperplastic foci are composed of type A and type B cells arranged in spherical nests or glandular structures. In the advanced stage, the edge of this lesion reaches the corticomedullary junction (Nyska and Maronpot, 1999).

This lesion is rare in young mice but has been reported to occur from 4 months old in both sexes. It has been reported to increase in incidence and severity with age and to be more common in aging females than males. Incidence may also increase in association with stressful housing conditions (Chvedoff et al., 1980).

The pathogenesis of subcapsular hyperplasia is thought to be related to hormonal alterations since gonadectomy or gonadal atrophy often enhances development in both sexes (Yoshida et al., 1986). Subcapsular hyperplasia may, but not inevitably, progress to adenoma (Dunn, 1970).

Differential diagnosis from subcapsular adenoma depends on the extent of the lesion; hyperplastic lesions can be focal, multifocal, or cicumferential. They do not extend into or beyond the capsule, and lesions are not larger than the normal width of the cortex in a young mouse. There is a lack of distinct compression; hyperplastic lesions may slightly bulge above the capsular surface and can cause minimal compression of the subjacent cortex (Capen et al., 2001).

Cortical Hyperplasia and Hypertrophy — Small focal lesions are occasionally found involving primarily the zona fasciculata of the adrenal cortex. There may be increased numbers of cells per unit area within the focus compared to the adjacent cortex (hyperplasia). These cells are smaller than normal cortical cells. Large foci of cortical hyperplasia are well circumscribed, blend with surrounding normal parenchyma, and cause no compression of adjacent cortex (Hamlin and Banas, 1990). It is not certain whether these represent true hyperplasia or an altered functional state resulting in a reduced cytoplasmic volume. Cortical hyperplasia is much less common than subcapsular cell hyperplasia (Nyska and Maronpot, 1999).

In other foci, the cells may be enlarged with abundant cytoplasm, which is eosinophilic or clear. Some foci contain both types of cells. They are usually single, focal, or less commonly diffuse lesions, which occur in the zona fasciculata and can extend into the zona glomerulosa.

Adrenal cortical hypertrophy is considered to reflect a functional change, maybe associated with stress and not a preneoplastic lesion (Nyska and Maronpot, 1999). Compression is minimal or nonexistent. These lesions must be distinguished from cortical adenomas, which cause distinct compression of adjacent tissue and have loss of normal architecture (Capen et al., 2001).

Medullary Hyperplasia — Single or, more rarely, multiple foci of small- to normal-size pheochromocytes with basophilic cytoplasm are occasionally found in the adrenal medulla. Foci of hyperplasia may occupy up to 50% of the normal size of the medulla. Medullary hyperplasia is considered to be part of the neoplastic continuum for adrenal medullary neoplasms (Nyska and Maronpot, 1999).

There is little if any compression of surrounding tissue, and the edges of the focus blend in with adjacent medullary parenchyma.

The main differential diagnosis is pheochromocytoma, which causes distinct compression at the periphery or loss of normal architecture (Capen et al., 2001). Hyperplasia and pheochromocytoma are the most common chemically induced adrenal lesions in the NTP in B6C3F1 mice.

Lipofuscin — Pigmented macrophages are commonly observed at the corticomedullary junction in aged mice of many strains, particularly females, and the incidence increases with age.

This lesion has also been referred to as ceroid deposition or "brown degeneration" (Dunn, 1970; Frith, 1983c). At first, the pigment appears as yellow-brown faintly granular material in the cortical cells adjacent to the medulla. As the lesion increases in severity, the cells become enlarged with abundant foamy brown to yellow cytoplasm and small pyknotic nuclei. Occasional multinucleate giant cells are found. The pigment is PAS positive and acid fast.

Other Nonneoplastic Lesions — Amyloidosis may be seen in the adrenal cortex, usually in the zona fasciculata (Sass, 1983b). Amyloidosis is discussed under the section on "Multisystem Diseases."

Hematopoiesis within the adrenal cortex is seen as part of a generalized response to hematopoietic stimuli in the animal.

Accessory adrenal cortical tissue is observed sporadically (Sass, 1983a).

Single or multiple spherical to ovoid nodules are located at one adrenal pole, within the cortex (subcapsular), or in the surrounding fat (Yarrington, 1996). Accessory nodules are subject to the same degenerative changes as the cortex.

They are best differentiated from cortical adenoma by lack of a common capsule, lack of capsular invasion, and normal cellular architecture (Nyska and Maronpot, 1999).

Cortical Vacuolation — Cells in the zona glomerulosa and zona fasciculata are characterized by variable cytoplasmic vacuolation, associated with their function of steroidogenesis from cholesterol.

Any toxin causing disruption of the cortex or enhancement of biosynthesis (via disturbance of the pituitary/hypothalamic hormonal regulation pathways) will therefore result in the accumulation of lipid-containing vacuoles (Dunn, 1970; Krinke et al., 2001b).

In females, the normal regression of the x-zone (located between the cortex and the medulla) appears as lipid vacuolization and should not be confused with a pathological process (Nyska and Maronpot, 1999).

Neoplastic Lesions

Subcapsular Cell Adenoma — These are benign tumors of old mice, composed of spindle-shaped (type A) cells comparable to those seen in subcapsular cell hyperplasia and causing compression of the adjacent adrenal cortex. Large polygonal type B cells are often found interspersed among the spindle cells, either singly or in nests. These tumors may be hormonally active, showing estrogenic (B cells), androgenic, or adrenocortical hormonal effects (Dunn, 1970).

Subcapsular Carcinoma — These malignant tumors tend to invade the capsule, show cellular pleomorphism, and frequent mitotic figures. Metastasis to the lungs is most common (Frith and Dunn, 1994).

Cortical Adenoma — Cortical B-cell adenomas consist of nodules of well-differentiated cortical cells that are demarcated from and compress the adjacent parenchyma (Frith, 1983a). There is distortion and loss of the normal cord arrangement. The cells are polygonal with abundant eosinophilic or amphophilic cytoplasm and round vesicular nuclei. There may be karyomegaly and cellular atypia. The presence of mitoses is variable.

The absence of invasion or distant metastases differentiates this lesion from cortical carcinoma.

Pheochromocytoma — Pheochromocytomas are tumors of the chromaffin (secretory) cells. These tumors tend to be unilateral in mice. Component cells are polyhedral with amphophilic or basophilic cytoplasm and basally located nuclei, or small and basophilic with little cytoplasm. The cells are arranged in trabeculae or nests separated by dilated vascular spaces. In the benign form, there is compression of adjacent tissue and loss of normal architecture. Cellular atypia and plentiful mitoses may be present. The size of the primary mass is reported to correlate best with the metastatic capability (Frith and Ward, 1988).

Malignant pheochromocytoma is diagnosed when there is evidence of capsular invasion or distant metastases. Subendothelial growth of tumor cells is common in adrenal medullary tumors and is not indicative of vascular invasion and malignancy. Similarly, small groups of medullary cells within the cortex, particularly in the region of the hilus, are a normal feature and should not be misinterpreted as evidence of malignancy.

Pheochromocytomas demonstrate a positive chromaffin reaction, staining with chromogranin A and tyrosine hydroxylase (Capen et al., 2001).

Ganglioneuroma is an infrequent tumor of the adrenal medulla, resembling nervous tissue containing large well-differentiated ganglion cells and neurofibrils. Diagnosis of malignant

ganglioneuroma is based on unequivocal evidence of invasion. These tumors stain positively with antibodies against synaptophysin and neuron-specific enolase (NSE; Capen et al., 2001).

Pituitary Gland

Nonneoplastic Lesions

Pituitary Cysts and Cystic Degeneration — True pituitary cysts, lined by epithelium, have been described in mice (Carlton and Gries, 1983). These cysts may be single or multiple, are usually microscopic and lined by ciliated cuboidal to columnar or infrequently by squamous epithelium, and contain eosinophilic colloid-like material. Those cysts close to or connected to the cleft between the pars distalis and intermedia are considered likely to be cystic remnants of Rathke's pouch.

Cystic or "cystoid degeneration" (Cameron and Sheldon, 1983) of the pars distalis is characterized by large spaces lacking a cyst wall but lined by viable pars distalis cells. Cystic spaces usually contain faintly granular eosinophilic material and occasionally cell debris. This lesion is seen as a spontaneous aging change or can be induced by estrogens. In addition, it is often seen as a component of proliferative changes in the pars distalis.

Proliferative changes in the pituitary gland (most commonly the pars distalis) are the most frequently observed spontaneous and induced lesions of the pituitary gland in mice (Frith and Ward, 1988). The incidence of spontaneous proliferative lesions increases with age. There is a biological and morphological continuum between hyperplasia, adenoma, and carcinoma of the pituitary gland.

Hyperplasia of the pars distalis may be focal, multifocal, or, rarely, diffuse. Focal hyperplasia is more common and more easily recognized. The cells comprising the focus are of a single cell type and may be any of the cell types found in the pars distalis. Most frequently, they resemble chromophobes, i.e., large pale cells with round nuclei. The borders of the foci blend into the adjacent parenchyma with little, if any, compression. Foci close to the periphery may cause slight elevation of the surface. Larger foci may contain areas of cystic degeneration or angiectasis. Differentiation from adenoma depends on the absence of compression and cellular atypia.

Neoplastic Lesions

Adenoma of the Pars Distalis — Adenomas of the pars distalis are common in female B6C3F1 and CD-1 mice and are infrequent in males. Adenoma of the pars distalis are solitary, sharply demarcated, expansile lesions causing distinct compression of the adjacent parenchyma. As with hyperplasia, they usually contain a single cell type. The cells are arranged in solid sheets or in cords, and large adenomas often contain areas of cystic degeneration or angiectasis. Cellular atypia may be present.

Carcinoma of the pars distalis is rare. Microscopic criteria for malignancy include vascular invasion, local invasion of the brain, or that of adjacent bone. Metastases are rare and, when they do occur, are usually intracranial.

Other Neoplasms — Occasional neoplasms of the pars intermedia have been reported (Goodman et al., 1981).

Thyroid Gland

Nonneoplastic Lesions

Cystic Follicles — Individual follicles may become enlarged and distended with colloid. These cystic follicles are lined by a single layer of cuboidal or flattened epithelium. Large follicles are normally found at the periphery of the thyroid.

Follicular Cell Hyperplasia — The lesion may be focal, multifocal, or diffuse. The affected follicles are of variable sizes. The epithelium is simple cuboidal to tall columnar and may form papillary projections into the lumen or form multiple small follicles within an enlarged follicle (cystic hyperplasia). Endocytosis of colloid may proceed at a greater rate than synthesis resulting in progressive depletion of colloid. The lack of compression of adjacent parenchyma helps to differentiate hyperplasia from follicular cell adenoma (Capen et al., 2001).

A number of goitrogenic compounds including thiourea, thiouracil, sulfa drugs, and aromatic amines exert their effect by inhibition of peroxidase-mediated incorporation of iodine into thyroglobulin. The resulting feedback mechanism causes increased secretion of TSH and follicular hyperplasia. Withdrawal of these compounds may result in the regression of the proliferative lesions; however, excessive TSH stimulation has also resulted in the progression of hyperplasia to neoplasia.

Other chemicals, such as phenobarbital and chlorinated hydrocarbon insecticides, act indirectly via the thyroid–pituitary axis. The initial change is the induction of hepatic cytochrome P450 enzymes. The resulting increased metabolism of thyroid hormone causes an increased rate of clearance of circulating T3 and T4, stimulating TSH secretion by the pituitary gland, and thyroid gland hyperplasia (Nyska and Maronpot, 1999).

Neoplastic Lesions

Follicular Cell Adenoma — Adenomas are discrete, well-circumscribed, lesions that often cause compression of the adjacent parenchyma. A capsule may or may not be present. These tumors may have papillary, follicular, or solid patterns (Heath and Frith, 1983). The cells are hyperchromatic, variable in size, and often have a high nuclear-to-cytoplasmic ratio. The cells are often multilayered, and nuclear crowding is common. The mitotic rate is usually low.

Follicular Cell Carcinoma — Carcinomas may have a papillary, solid, or follicular pattern or combinations thereof (Heath and Frith, 1983). The lesion is often at least partially encapsulated with a dense scirrhous reaction and neoplastic cells or follicles within the capsule. The cells are pleomorphic with a high nuclear-to-cytoplasmic ratio. The mitotic rates are variable but often high. Invasion of adjacent structures is common.

Other Neoplasms — C-cell adenomas and carcinomas are extremely uncommon in mice (Frith and Heath, 1983a,b; Russfield, 1982; Squire et al., 1978; Van Zwieten et al., 1983).

Parathyroid

Spontaneous lesions, both neoplastic and nonneoplastic, are uncommon in mice (Russfield, 1982; Squire et al., 1978).

Diffuse bilateral hyperplasia may occur in response to renal disease (renal secondary hyperparathyroidism). Focal hyperplasia is usually unilateral and is not associated with increased parathyroid hormone secretion (Capen et al., 2001).

Pancreatic Islets

Nonneoplastic Lesions

Islet Cell Hyperplasia — Certain mouse strains have high incidences of this lesion (Sass et al., 1978). Hyperplasia of the pancreatic islets usually involves more than one islet (multifocal) and may involve all islets visible in a histologic section. The islets are much enlarged owing to an increased number of cells, which are morphologically similar to those in smaller normal islets. There is no

encapsulation or compression of adjacent acinar tissue, and islet architecture is generally maintained. All of these features help differentiate this lesion from islet cell adenoma. The specific cell type involved is difficult to identify at the light microscopic level, and histochemistry and electron microscopy are often needed. Immunoperoxidase staining of the hyperplastic islets has revealed that most of the cells contain insulin and some contain somatostatin.

Neoplastic Lesions

Islet Cell Adenoma — Islet cell adenomas in mice commonly involve a single islet within a histological section (Frith and Sheldon, 1983), are larger than hyperplastic islets, and compress adjacent normal pancreas. The cells form ribbons along sinusoidal, thin-walled vessels, and the adenomas often appear more vascular than hyperplastic islets. The cells stain lightly eosinophilic with H&E; the nuclei demonstrate a delicate chromatin pattern. The cells are well differentiated, and mitotic figures are few in number.

Islet Cell Carcinoma — Islet cell carcinomas are invariably larger than adenomas and are commonly visible grossly. The cells vary from well-differentiated cells to extremely pleomorphic and anaplastic cells (Frith and Sheldon, 1983). Well-differentiated islet cell carcinomas usually invade locally and occasionally metastasize. Most tumors are probably insulinomas, but do not cause hypoglycemia. The cytoplasm of the neoplastic cells is eosinophilic, and the nuclei are vesicular. Nucleoli are prominent and may be multiple. Mitotic figures are evident, and pleomorphism may be prominent. Some anaplastic carcinomas may be difficult to classify with certainty as islet cell in origin.

Female Genital System

Ovary

Nonneoplastic Lesions

Ovarian Cysts — Cysts of the ovary and paraovarian tissues are extremely common in aged females of most strains of mice (Burek et al., 1982; Frith and Ward, 1988; Goodman et al., 1981). They may be focal or multifocal and may become quite large, compressing and replacing most of the ovary in some instances. The character of the lining cells varies depending on the etiology of the cyst. There are often foci of chronic inflammatory cells in the capsule. Pigment-laden macrophages are also common. The cysts may be filled with clear fluid or may be hemorrhagic.

Ovarian cysts may originate from several different sites and comprise follicular and luteal cysts, epithelial inclusion cysts, epidermoid cysts, paraovarian cysts, rete cysts, and bursal cysts (Davis et al., 1999).

Follicular and luteal cysts: these are both derived from anovulatory Graafian follicles. Follicular cysts are lined by cuboidal granulosa cells, whereas luteinized follicular cysts are lined by luteinized granulosa cells with vacuolated cytoplasm.

Epithelial inclusion cysts are lined by columnar epithelial cells that form papillary structures. These cysts are age related and also considered to be precursor lesions of epithelial adenomas and carcinomas.

Epidermoid cysts are lined by squamous epithelial cells and are filled with layers of keratin. They are frequently found in association with teratomas.

Paraovarian cysts originate from the mesovarium and are lined by ciliated columnar epithelial cells. Smooth muscle is present in the wall of the cyst.

Rete Cysts — Rete ovarii are composed of tubules lined by columnar epithelium. Cysts of the rete ovarii are morphologically similar to paraovarian cysts but do not contain smooth muscle.

Bursal cysts are commonly found lesions that may cause compression of the ovary.

Atrophy — This occurs in adult mice and is characterized by decreased numbers of follicles at all stages of maturation and of corpora lutea. There is an increase in the interstitial tissue.

Inflammation — Acute inflammation with abscess formation is rare in mice but does occur, and *Klebsiella* has been cultured from some of these cases.

Lipofuscin — Lipofuscin is common in the ovarian stroma of aged female mice. Lipofuscinosis is characterized by large round cells with abundant, foamy, pale yellow, pigmented cytoplasm that are present in the ovarian stroma. The pigment is acid fast and PAS positive.

Hyperplastic Lesions

Tubular Hyperplasia/Epithelial Hyperplasia — Epithelial hyperplasia is characterized by groups of elongate tubules lined by cuboidal/columnar epithelial cells that dissect through the ovarian stroma. The cells have small round nuclei and scant cytoplasm. Epithelial hyperplasia is an age-related change and has also been chemically induced by 1,3 butadiene and 4-vinylcyclohexene. These chemicals induce both hyperplasia and neoplasia of the ovarian epithelium.

Interstitial Cell (Stromal/Luteal) Hyperplasia — The lesion consists of enlargement and increased numbers of interstitial cells. The cells contain abundant foamy cytoplasm and are arranged in nests and packets by delicate stromal fibers.

Neoplastic Lesions

Tubulostromal Adenoma — These neoplasms arise by the downgrowth of the germinal epithelium into the ovarian stroma. The tubules are similar to those seen in epithelial hyperplasia. The tubular structures replace the entire ovary and occasionally invade paraovarian fat (Carter, 1968; Frith and Ward, 1988; Goodman et al., 1981; Goodman and Strandberg, 1982; Morgan and Alison, 1987b). One of the distinguishing factors from tubular hyperplasia is that the diameter of proliferative lesion is larger than a corpus luteum.

Tubulostromal Adenocarcinoma — These malignant tumors show a high degree of pleomorphism, atypia, and an infiltrative growth pattern (Davis et al., 2001).

Cystadenoma — Ovarian cystadenomas are seen sporadically in many strains of mice (Carter, 1968; Frith and Ward, 1988; Frith and Wiley, 1981; Goodman et al., 1981; Goodman and Strandberg, 1982; Morgan and Alison, 1987a). Cystadenomas are the most common age-related ovarian neoplasm found in studies in the female B6C3F1 mouse (Alison et al., 1987). Microscopically, they are cystic tumors lined by cuboidal to columnar epithelium with basal nuclei. There are usually simple to complex papillary structures projecting into the lumen and lined by similar epithelium.

Cystadenocarcinoma — The cytology of these neoplasms is more atypical than cystadenomas with an increased mitotic activity.

Granulosa Cell Tumors — These are the most commonly chemically induced neoplasm in the NTP 2-year carcinogenicity studies and may be either benign or malignant (Alison et al., 1987).

They are composed of variably sized follicles, which may cause considerable compression of the adjacent ovary. The cells resemble granulosa cells of normal follicles. The hallmark of malignant granulosa cell tumors is invasion and metastasis. The cells of malignant tumors are more pleomorphic than those of benign tumors with high mitotic activity. Occasionally, granulosa cell tumors show areas of fusiform theca-like cells.

Thecoma, Benign — These neoplasms are composed of densely packed fusiform cells arranged in interlacing bundles and whorls. There is a variable amount of lipid and collagen present.

Thecoma, Malignant — These differ from the benign tumors in that they have extensive areas of necrosis, pleomorphism infiltration of adjacent tissue, and/or distant metastases.

Luteoma — Luteomas are composed of large polygonal cells with abundant pale cytoplasm and round central nuclei. The cells are arranged in nests and cords. The tumors are generally well circumscribed, although not encapsulated, and often involve the greater portion of the ovary (Carter, 1968; Frith and Ward, 1988; Goodman et al., 1981).
     These tumors are the most common type of ovarian tumor seen in the CD-1 mouse.

Yolk Sac Carcinomas — These are rare tumors in the mouse. A diagnostic feature of these neoplasms is that the tumor cells produce an abundant eosinophilic PAS-positive matrix in which nests and cords of neoplastic cells are embedded.

Vascular Tumors — Hemangiomas are more commonly diagnosed than hemangiosarcomas. The former are difficult to distinguish from angiectasis.

Other Neoplasms — Both benign and malignant teratomas are seen occasionally in mice. Dysgerminomas and Sertoli cell tumors are extremely rare (Alison et al., 1987; Alison and Morgan, 1987; Carter, 1968; Frith and Ward, 1988).

Uterus, Uterine Cervix

Nonneoplastic Lesions

Cystic Endometrial Hyperplasia — This lesion is extremely common in aged female mice (Burek et al., 1982; Frith and Ward, 1988; Goodman et al., 1981). There are both proliferation and dilatation of the endometrial glands. The glands are lined by columnar epithelium, and the lumens are often filled with eosinophilic material. In severe lesions, large cysts may develop. These are lined with low cuboidal or flattened epithelium.

Hydrometra — Marked dilatation of the lumen of the uterus with fluid is termed hydrometra. The wall of the uterus is thinned, and there is loss of the endometrial glands. The lumen is filled with faint fibrillar eosinophilic fluid or mucoid material.

Endometrial Stromal Hyperplasia — This is a frequent finding in the mouse cervix and uterus. It is characterized by a proliferation of stromal cells with a variable amount of collagen. The growth characteristics of these lesions are noninvasive. The lesion is generally oriented along the normal anatomic structures, e.g., circular around the cervix, and does not cause any great distortion of anatomic structures.

Neoplastic Lesions

Endometrial Stromal Polyp — These neoplasms occur frequently in many strains of mice (Goodman and Strandberg, 1982). They are small masses that project into the uterine cavity. Microscopically, the mass consists of an edematous stroma composed of spindle-shaped or stellate cells and varying numbers of endometrial glands, which may be cystic. The stroma is often highly vascular, and the surface is covered by a single layer of simple columnar epithelium.

Endometrial Adenoma — These tumors arise from the epithelium lining the uterine mucosa or that of the endometrial glands. The epithelial cells are arranged in papillary, glandular, or tubular structures.

Endometrial Adenocarcinoma — These malignant tumors are composed of epithelial cells arranged in acini, glandular structures, and in solid nests and cords. The neoplastic glands may show varying degrees of pleomorphism and atypia.
    Endometrial adenocarcinomas are invasive and may metastasize, especially to the lungs.

Endometrial Stromal Sarcoma — These are the most commonly observed uterine tumors in aged B6C3F1 mice. These neoplasms arise in the endometrium and are composed of sheets of spindle cells with scant pale eosinophilic cytoplasm and elongated, hyperchromatic, fusiform nuclei. Cell borders are usually indistinct. The cells may be organized in fasciculi, which run at angles or perpendicular to one another and in which the nuclei, cut in cross-section, may appear round or oval. Occasional areas may be present in which the fusiform nuclei appear plump and contain vacuoles. Areas of necrosis are common. Cellular atypia and mitotic figures may be present but are variable. Spread is by infiltration into the myometrium, cervix, and serosa. Metastasis is infrequent. It is necessary to distinguish endometrial stromal sarcoma from mesenchymal tumors such as leiomyosarcoma or fibrosarcoma (Goodman and Strandberg, 1982) as well as histiocytic sarcoma.

Leiomyoma — Leiomyomas are benign tumors of smooth muscle that are well circumscribed. They are composed of interlacing compact bundles of fusiform smooth muscle fibers that frequently run perpendicular to each other. The nuclei are elongate with round to blunt ends and are centrally located. These tumors are seen more frequently in CD-1 mice than in B6C3F1 mice.

Leiomyosarcoma — These neoplasms are malignant tumors of smooth muscle. They infiltrate the uterine wall and invade through the serosa. The cells are less well-differentiated smooth muscle cells than those seen in leiomyomas and are arranged in interlacing or whorling patterns. Mitotic activity is variable. Smooth muscle tumors stain positively for desmin and smooth muscle actin.

Squamous Cell Carcinoma — This is a rare spontaneous tumor but may be readily induced by estrogens.
    This tumor is usually well differentiated and is heavily infiltrated by leukocytes.

Granular Cell Tumor Benign — The origin of these tumors is not established but thought to be either from Schwann cells or from mesenchymal cells. These are solid masses with large epithelioid round/oval cells with large pale nuclei and abundant eosinophilic cytoplasm.

Vagina

    Spontaneous lesions of the vagina are rare in mice of all strains (Frith and Ward, 1988; Goodman and Strandberg, 1982).

Cervical and vaginal granular cell neoplasms have been diagnosed in B6C3F1 mice. These tumors are composed of sheets of large round or polygonal PAS-positive cells with an intervening fine fibrovascular stroma.

Induced Neoplasms

Squamous cell carcinomas are induced by AZT and estrogens.

Clitoral Gland

The clitoral gland is a skin appendage composed of modified sebaceous glands.

Nonneoplastic Lesions

Cystic Ducts (Ectasia) — Dilatation of the ducts of the clitoral glands occurs frequently and may be associated with inflammatory changes, either acute or chronic.

Neoplastic Lesions

Neoplasms of the clitoral gland are very uncommon, but they may be chemically induced.

Acinar Cell Adenomas — These benign tumors are characterized by neoplastic acini composed of pale foamy sebaceous-type cells with peripheral basophilic basal cells. The cytoplasm may contain eosinophilic granules, and there may be squamous metaplasia.

Acinar Cell Adenocarcinoma — These malignant tumors are composed of variably sized nests and nodules of proliferating cells. The acinar structures are composed of pale foamy sebaceous-type cells with peripheral, small, basophilic basal cells.
There may be areas of squamous differentiation, cystic areas, necrosis, and inflammation. There is evidence of local invasion.

Squamous Cell Papilloma — These benign tumors are composed of papillary structures with a central connective tissue stalk that is lined by a squamous epithelium, which may or may not be keratinized.
These tumors may be single or multiple and may obstruct the ducts.

Squamous Cell Carcinoma — These malignant tumors consist of irregular papillary fronds or nodules of pleomorphic squamous cells with local invasion. The amount of keratinization is variable. Mitotic figures, necrosis, and inflammation are commonly present.

Mammary Gland

The development of mammary tumors in mice is influenced by many factors. These include genetics, an oncogenic retrovirus (murine mammary tumor virus infection: MuMTV), age, sex, diet, hormonal status, and immune status (Seely and Boorman, 1999).
Strains with MuMTV have a high incidence of spontaneous mammary gland tumors, e.g., C3H and DBA/Z. Strains that are either free of MuMTV or genetically resistant such as B6C3F1, Balb/C, and C57Bl mice have a low incidence of spontaneously occurring mammary tumors.
In susceptible mice, MuMTV is passed on from generation to generation by one of two ways either via infected germ cells or through the milk during lactation. There are several distinct lesions associated with MuMTV infection such as hyperplastic alveolar nodules (HAN) and plaques. These are both considered to be preneoplastic lesions although there is no certain progression to

malignancy. HAN consist of proliferating acini, are transplantable only to mammary fat pads, and are found in nonpregnant and nonlactating mice. Carcinomas may develop within HAN. Plaques are pregnancy dependent and are branching tubules radially oriented in loose connective tissue.

There is multiplicity of endocrine influences on both mammary gland development and tumorigenesis. Hormones produced by ovaries, pituitary, adrenals, and placenta have an important permanent effect on mammary gland development.

Nonneoplastic Lesions

Spontaneous nonneoplastic lesions of the mammary gland are uncommon in B6C171 and CD-1 mice. Ectasia of mammary ducts are occasionally observed.

Hyperplasia — This change may be a focal or diffuse change. Physiological hyperplasia is a feature of late gestation and during lactation.

Focal hyperplasia is characterized by normal-appearing alveoli and ducts that are increased in number. There is no cellular pleomorphism, compression, or encapsulation. The regular glandular architecture of the mammary gland is maintained (Bruner et al., 2001).

Neoplastic Lesions

Adenomas — These benign tumors are composed of well-demarcated proliferations of small solid acinar structures that are uniform in size and closely packed. There is scanty intervening connective tissue. The cells are well differentiated, and the epithelial lining of the acinar structures is single layered. The regular lobular architecture of the mammary gland is not maintained.

Adenocarcinomas — These malignant tumors differ from adenomas in that there is a high variability in growth pattern with papillary, tubular, cystic, solid, comedo, and undifferentiated morphologies evident. Typically, mammary adenocarcinomas contain variably sized cystic structures lined by pleomorphic or frankly anaplastic cuboidal acinar epithelium. Mitoses are frequently observed. There is either local invasion of adjacent tissues or metastasis. There is frequently necrosis and hemorrhage. There is a variable amount of stroma present. These tumors are usually large. Hemangioendothelial cell hyperplasia in the heart in female B6C3F1 mice has been reported in mice bearing mammary adenocarcinomas (Iwata et al., 1994).

Fibroadenoma — These are benign tumors that are clearly demarcated with alveoli or ducts present within a prominent dense fibrous stroma. The amount of connective tissue enclosing glandular structures is variable. There is usually a fibrous capsule.

Adenoacanthoma — Despite its title, this tumor is malignant. It is typically a well-circumscribed tumor comprising glandular and squamous epithelial elements that are present in variable proportions (25% or more of the tumor consists of squamous metaplasia). The squamous cells exhibit intracytoplasmic keratin and pearl formation. Metastases may exhibit either squamous or glandular tissue. These tumors have a high incidence in the BALB/C strain.

Squamous Cell Carcinomas — These are rare tumors that have been chemically induced.

Induction of Mammary Tumors in Mice

Mammary tumors in mice have been induced by many chemicals. In the NTP program, chemicals that have induced (mainly malignant) mammary tumors in female mice include benzene,

1,3-butadiene, 1,2-dibromoethane, glycidol, sulfallate, chloroprene, 1,2-dichloroethane, benzene, furosemide, ethylene oxide, and reserpine (Dunnick et al., 1995).

Factors such as the presence of MuMTV, the hormonal balance, and immune status influence the development of chemically induced neoplasms. In butadiene-induced adenocarcinomas, several tumor suppressor genes were reported to be inactivated (Wiseman et al., 1994). The p53 pathway may be a common target for mutation in mammary gland tumor carcinogenesis (Jerry et al., 1993).

Hematopoietic System

Nonneoplastic Lesions

Thymus

Thymic Cysts — Thymic cysts have been seen in a number of strains of mice, and they generally increase in incidence with age (Frith and Wiley, 1981). The cysts are lined by a simple cuboidal epithelium, and the lumen is usually empty. In some strains, they may occur in the cranial part of the thymus at the corticomedullary junction (Wijnands et al., 1996; Yoshida et al., 1986).

Thymic Atrophy — Histologically, the appearance of thymic atrophy and normal involution is similar, but atrophy is seen in young mice, in response to a variety of toxic insults and is reversible on removal of the stimulus, and involution is seen in older mice and is irreversible (Ward et al., 1999).

Atrophy begins as a depletion of cortical lymphocytes, with loss of cortico-medullary differentiation, progressing to more generalized depletion, and all cell types are decreased in number.

Apoptosis is seen in physiological involution and at an increased level in association with intoxication, infection, or injury. The primary mechanism of the induced change has been shown to be stress, mediated by glucocorticoids (Levin, 1998; Levin et al., 1999). Atrophy can also be produced as a result of direct toxicity and decreased levels of growth hormone (Gopinath et al., 1987) and administration of immunosuppressive agents such as corticosteroids, cyclosporine, and cyclophosphamide (Greaves, 1990; Schuurman et al., 1991).

Ectopic Thymus — Ectopic thymus has been reported adjacent to or in association with the parathyroid gland. The ectopic tissue is predominantly cortical thymic tissue.

Conversely, ectopic parathyroid tissue can be seen close to or embedded within the thymus (Frith and Fetters, 1983). This association is due to the simultaneous migration of these tissues early in gestation.

Spleen

Accessory Spleen — A small accessory spleen is occasionally noted in mice. The accessory spleen is usually near the normal spleen and may be embedded in the pancreas. Normal red and white pulp is usually present.

Splenic Pigmentation — Hemosiderin, ceroid or lipofuscin, and melanin pigment may occur in the spleens of mice.

Hemosiderin is a golden brown granular pigment derived from the breakdown of RBC and present within the cytoplasm of macrophages. It stains positively with iron stains such as Prussian blue. Hemosiderin is found, to some extent, in mice of all ages, is generally more prominent in females than males, and increases in amount with age. Background levels reflect the normal removal of effete RBC by the spleen, but hemosiderin can be increased in cases of chemically induced hemolytic anemia or methemoglobinemia (Travlos et al., 1996).

Ceroid or lipofuscin pigment is another golden brown pigment seen within the cytoplasm of macrophages in the spleen. It is derived from oxidation and polymerization of fatty acids and stains positively with acid-fast stains (Crichton et al., 1978).

Melanin pigment may occur in the spleen of mice with pigmented skin. It is slightly darker than hemosiderin and has a characteristic elongated or stringy appearance. It is not associated with macrophages, being present in melanocytes, and is iron negative.

Extramedullary Hematopoiesis — Hematopoiesis is normally carried out in the fetal and neonatal livers. In the adult, this function is taken over by the spleen and bone marrow (Harada et al., 1996).

Extramedullary hematopoiesis (EMH) is therefore often seen within the splenic red pulp in mice (Long et al., 1986) and can be increased in response to a number of stimuli such as inflammation, anemia, immune stimulation, cytokine effects, thrombocytopenia, and certain neoplasms. Foci of EMH are composed of variable numbers of megakaryocytes, myeloid, and erythroid precursors. There may also be an associated plasma cell hyperplasia. The predominant cell type present will depend on the nature of the initiating stimulus.

Hemorrhage or anemia results in erythropoiesis. Erythropoietic activity is characterized by foci of immature erythrocytic precursors with small darkly staining nuclei in the red pulp. It may or may not be associated with an increase in the granulopoietic activity.

Hyperplasia of granulocytic elements is usually accompanied by initiating lesions such as abscesses, ulcerative tumors, or other inflammatory lesions.

A generalized increase in granulopoiesis may be accompanied by granulopoietic elements in a variety of other organs, including the liver, adrenals, and lymph nodes. This is also referred to as a leukemoid reaction.

A marked increase in granulopoietic activity must be distinguished from granulocytic leukemia. In granulopoiesis, the complete series of developing cells, including the mature neutrophils, is usually present. In granulocytic leukemia, the developing stage of the neoplastic granulocytes may vary from animal to animal, but typically a single stage predominates (Dunn, 1954).

Lymphoid Hyperplasia — Hyperplasia of a variety of cell types may occur in hematopoietic tissue of mice.

Lymphoid hyperplasia in the spleen is more common in females than males and can have a variety of morphological patterns: involving germinal centers, marginal zones, or white pulp, each of which may occur concurrently (Ward, 1990b).

Differentiation from lymphoma depends largely on the maintenance of normal architecture, involvement of multiple follicles, confinement to the spleen, and demonstration of the cause for the reactive change, e.g., inflammation (Frith et al., 2001).

Classification of hyperplastic lesions in the lymph nodes of rodents has been described (Ward, 1990a).

Lymphoid hyperplasia of the lymph nodes is common, but it is rare in mice under 12 months of age. The incidence increases slightly with age and is slightly greater in females than in males in most strains. In mice, active hyperplasia is often seen in the mandibular and mesenteric nodes.

Hyperplasia may be seen in the B-cell areas (follicles, germinal centers), T-cell thymic-dependent areas (paracortex), and medullary cords or sinuses. Commonly in the hyperplastic lymph node, the normal corticomedullary division is not apparent (paracortical hyperplasia), and the marginal sinus is filled with lymphocytes. The lymphocytes are usually small and normal in appearance.

The lesion is difficult to distinguish from the lymphoblastic type of malignant lymphoma if only a single node is involved; but the lymphocytes are mature, few mitotic figures are observed, and the "starry sky" effect found in lymphoblastic lymphomas is not seen. Thickening of the medullary cords is often noted and is characterized by the presence of many plasma cells (plasmacytosis),

occasionally resembling plasmacytoma. The plasmacytosis is a reaction to chronic inflammatory lesions or tumor antigens. It is most commonly seen in the mandibular nodes.

Lymph Nodes

Sinus Histiocytosis — Accumulations of normal histiocytes may occur in the subcapsular and medullary sinuses of lymph nodes. The histiocytes have dark basophilic nuclei and abundant distinctly eosinophilic cytoplasm. They may contain hemosiderin and other pigments, erythrocytes, and other phagocytized material.

Plasmacytosis — Plasmacytosis is an increased number of normal-appearing plasma cells in lymph nodes. The plasmacytosis may be a reaction to an adjacent chronic inflammatory lesion or to tumor antigens. The most common lymph node involved is the submandibular lymph node.

Mastocytosis — An increase in mast cells (mastocytosis) may occur in either the splenic red pulp or lymph nodes. Differentiation of this lesion from metastatic mast cell tumor depends on the identification of the primary mast cell tumor.

The number of mast cells normally found in a specific organ may vary from strain to strain (Dunn, 1969).

Miscellaneous Lesions — Megakaryocytosis, bone marrow atrophy, necrosis, atrophy, and mineralization of lymph nodes may occur in mice, but all are rare in both the CD-1 and B6C3F1 strains.

Neoplastic Lesions

Malignant Lymphoma

Malignant lymphomas arise from B or T lymphocytes or their precursors (Frith et al., 2001). Those tumors of B-cell origin are follicular center cell (FCC), plasma cell, and immunoblastic lymphoma. Plasmacytic lymphomas originate from immunoblasts.

The cell membrane antigen CD45R is seen on the surface of normal and neoplastic B cells, and therefore can be a useful marker in differentiating the cell of origin; however, the intensity of immunoreactivity may be decreased in tumor cells. Immunoglobulin stains (e.g., IgG and IgA) are useful markers for immunoglobulin-producing B cells (plasma cell and immunoblastic lymphoma).

Others such as small lymphocyte lymphomas may be of B- or T-cell origin. Lymphoblastic lymphoma can also be of B- or T-cell origin but usually arise from T cells in the thymus.

T-cell tumors most often arise in the thymus and metastasize to other tissues including the spleen (Dunnick et al., 1997; Hursting et al., 1995). They are commonly CD3 immunoreactive.

The immunomorphological classification illustrated in Table 2.23 is adapted from Pattengale and Frith (1983).

Follicular Center Cell Lymphoma

Reticulum cell sarcoma type B as described by Dunn (1954) has been referred to as malignant lymphoma, mixed-cell type (Frith and Wiley, 1981) or pleomorphic cell type, and more recently has been identified as a FCC lymphoma of B-cell origin (Frederickson et al., 1985; Pattengale and Frith, 1983). The B-cell nature of these cells has been confirmed using immunoperoxidase techniques with Bouin's or B-5 fixed tumors demonstrating the presence of cytoplasmic or cell surface immunoglobulin (CIg) of the various isotypes.

Table 2.23   **Immunomorphological Classification of Murine Lymphomas, Related Leukemias, or Tumors**

Morphological Type	Immunological Type			
	B Cell	T Cell	Non-B, Non-T	Histiocyte
Follice center cell				
Small cell type	+	−	−	−
Large cell type	+	−	−	−
Large and small (mixed) cell type	+	−	−	−
Plasma cell	+	−	−	−
Immunoblast	+	(+)[a]	(+)	−
Small lymphocyte	+	(+)	(+)	−
Lymphoblast	+	+	+	−
Histiocyte	−	−	−	+

Source: Adapted from Pattengale, P. K. and Frith, C. H., *J. Natl. Cancer Inst.*, 70, 169.
[a] Parentheses indicate that the disease has been seen in humans, but to date has not been reported in the mouse.

Most lymphomas in B6C3F1 mice are of FCC origin, based on antigenic staining and molecular studies (Della Porta et al., 1979).

The disease is rare before 12 months of age and may increase dramatically in some strains after 18 months. This neoplasm is slightly more common in female than in male mice. Lesions often arise in individual mesenteric lymph nodes, intestinal Peyer's patches, or in one or more germinal centers of the follicles in the B-cell areas of the white pulp.

Microscopically, FCC lymphomas express varying degrees of pleomorphism. They may be composed of small FCCs (centrocytes), large FCCs (centroblasts), or most commonly a mixture of the two small lymphocytes, macrophages, and immunoblasts. The proportion of each cell type varies with each lymphoma.

In lymphomas composed of large FCCs, the cells are large and cohesive with irregularly shaped folded and notched nuclei (cleaved) and moderate amounts of cytoplasm. Small FCCs are cohesive, and nuclei are markedly irregular in size and shape with scant cytoplasm.

Immunoblastic Lymphoma

Immunoblastic lymphoma of B-cell origin is rare in the mouse (Pattengale and Frith, 1983). The pattern of organ involvement in immunoblastic lymphoma is similar to that of FCC lymphoma but is usually more invasive in its behavior (Ward et al., 1999). It is characterized by noncohesive, large lymphoid cells with round-to-oval vesicular nuclei with prominent and distinct nucleoli. In addition, the nuclei are sometimes eccentric and have clumped, peripherally marginated clock-face-like chromatin, and the cytoplasm is moderately dense and amphophilic (plasmacytoid features).

Plasma Cell Lymphomas

Plasma cell lymphomas occur infrequently in control mice. The lymph nodes, spleen, and liver may be involved. The cells are large with amphophilic cytoplasm, some are binucleate, the mitotic index is high, and the cells retain a characteristic plasma cell appearance. Spontaneous plasma cell myelomas are rare, but ip plasma cell tumors can be induced with ip injections of mineral oil.

Lymphoblastic Lymphoma

Using immunocytochemical techniques and the newer classification of murine malignant lymphomas (Frederickson et al., 1985; Frith et al., 1985; Pattengale and Frith, 1983), the lymphoblastic type is comparable to Dunn's lymphocytic leukemia. This is the most common hematopoietic tumor in the CD-1 mouse and is one of the more common hematopoietic neoplasms in many other strains. T-lymphoblastic lymphoma may occur as early as 1 month of age and may peak at 3–6 months in some strains such as the BALB/c and AKR mice. The incidence is generally higher in female than in male mice.

Lymphoblastic lymphoma often arises in the thymus (T lymphoblastic) but is aggressive involving a number of organs, most commonly the liver, spleen, lymph nodes, bone marrow, and lungs. In the lungs, invasion follows the vascular tree. It is often leukemic and may involve the central nervous system. The component cells are medium-sized lymphoblasts with high nuclear-to-cytoplasmic ratio, moderate amounts of basophilic cytoplasm, which may be vacuolated, and round vesicular nuclei, with prominent central nucleoli. Mitoses are numerous.

Small Lymphocyte Lymphomas

Component cells are well-differentiated small-to-medium-sized lymphocytes, which are morphologically indistinguishable from normal circulating lymphocytes. Cells are uniform and noncohesive, and mitotic figures are rare. Tingible-body macrophages are not present, and there is effacement of normal tissue architecture.

Other Neoplasms

Histiocytic Sarcoma — Dunn's (1954) reticulum cell neoplasm type A, or malignant lymphoma (Frith et al., 1981), histiocytic type, or histiocytic lymphoma, has been recently classified as histiocytic sarcoma (Pattengale and Frith, 1983). Other investigators have described the lesion as endometrial sarcoma or malignant schwannoma (Stewart et al., 1974). Research findings have suggested that the neoplastic cells are derived from histiocytic cells of uncertain origin (tissue histiocytes, Kupffer's cells, macrophages) (Frith et al., 1981).

Histiocytic sarcomas are common in both CD-1 and B6C3F1 mice over 12 months old and are slightly more common in females than in males. The liver is the most commonly involved organ in male mice; in females, the uterus and vagina as well as the liver are often involved, suggesting these tissues as sites of origin. Other organs less frequently involved include the spleen, lymph node, bone marrow, lung, kidney, and ovaries. Metastatic lesions to the lungs occur in a high percentage of cases with liver involvement. Component cells are small spindle-shaped to round cells with small dark nuclei and scant-to-moderate amounts of eosinophilic cytoplasm. Multinucleate forms are scattered throughout the tumor. There are frequently zones of necrosis within these tumors.

Thymoma — The term thymoma is usually used in the mouse to classify a lesion characterized by the presence of a neoplastic epithelial component with or without neoplastic lymphocytes. The epithelial component is positive for keratin and appears to be derived from the epithelial cells in Hassall's corpuscles or thymic reticular tissue. This type of tumor is rare in all strains of mice.

Benign thymomas are solitary lesions that may be well encapsulated. In the malignant form, there is marked local invasion beyond the confines of the thymus.

Other rare nonlymphoid hematopoietic neoplasms in the mouse include mast cell tumor (Deringer and Dunn, 1947; Dunn, 1969; Frith and Dooley, 1976), granulocytic leukemia (Dunn, 1954; Frith et al., 1981), and erythroleukemia (Frith et al., 1990).

Male Genital System

Testes

The testicular germinal epithelium is sensitive to a wide variety of internal and external influences (Faccini et al., 1990c). These include age, nutrition, hormones, temperature, lighting, stress, vascular perfusion, and spaceffects. Effects on other cell components of the testis, for example, Sertoli cells, may have a knock-on effect of secondarily causing degeneration of germinal epithelium.

Nonneoplastic Lesions

Hypospermia — Hypospermia (immaturity of the seminiferous tubules) is the normal condition in very young mice (<4 weeks of age), but it may be induced in older mice with toxicants. Maturing and mature spermatozoa are absent from both the testes and the epididymis.

Testicular Atrophy — Testicular atrophy occurs as an aging lesion in mice and may also be due to other factors such as exposure to specific toxic compounds, irradiation, and hypoxia. The lesion may be focal or diffuse. The seminiferous tubules and germinal epithelial cells are reduced in number, and the testis may also be reduced grossly in size. A relative increase in Sertoli cells, interstitial cells, or multinucleated cells may be seen. The lipofuscin pigment ceroid may be associated with the atrophy. Ceroid is acid fast and PAS positive.

Testicular Mineralization — Focal dystrophic mineralization of the seminiferous tubules may occur occasionally. It may represent previous areas of injury. The mineralization is composed of basophilic concentric masses, which may be amorphous or concentrically laminated.

Hyperplasia of Interstitial (Leydig) Cells — An increase in the relative number of interstitial cells may be associated with testicular atrophy. Focal hyperplasia of interstitial cells may also occur and possibly represents a preneoplastic condition. Diffuse hyperplasia precedes tumor formation in mice with testicular feminization and is associated with estrogenic compounds and five gamma reductase inhibitors (Radovsky et al., 1999).

"Spermatocele" can occur in any part of the ductular system seminiferous tubules or epididymis and consists of a dilation of the lumen by accumulation of spermatozoa.

"Sperm granulomas" result from the rupture of the seminiferous tubules or passages conducting spermatozoa. The spermatozoa elicit an inflammatory response because they are coated with proteins that incite an immune response. The granulomatous response may be so florid that it is important to differentiate it from a neoplasm.

Neoplastic Lesions

B6C3F1 mice have an overall low incidence of neoplasms in male reproductive organs (Mitsumori and Elwell, 1988).

Interstitial Cell Tumors — Both benign and malignant spontaneous tumors of the interstitial cells of Leydig are rare in most strains of mice, but can be seen in both the CD-1 and the B6C3F1 mouse. Neoplasms can be induced with synthetic or natural estrogens in certain strains, particularly BALB/c mice. The cytoplasm of the neoplastic cells is distinctly eosinophilic. Small, well-circumscribed tumors are designated adenomas, and large tumors that are invasive or metastasize are referred to as carcinomas. The larger carcinomas occasionally metastasize to the lungs.

Sertoli Cell Tumors — "Sertoli cell tumors" of the testis have been described although they are extremely rare in mice (Rehm et al., 2001). The characteristics of these neoplasms are areas of palisading cells within poorly demarcated tubular structures. The tumor cells are elongated with central nuclei in abundant pale eosinophilic cytoplasm.

Gonadal stromal tumors (these tumors resemble ovarian granulosa cell tumors). Cells are in nests cords or sheets and occasionally in follicular patterns. Cells have scant amphophilic cytoplasm and distinct nuclei.

Other tumors arising within the testis are teratomas, seminomas, yolk sac carcinomas, and adenomas and carcinomas of the rete testis. These are all rare neoplasms.

Accessory Sex Glands

Nonneoplastic Lesions

Prostate — Foci of chronic inflammatory cells are a frequent finding in the interstitial tissue of the prostate.

Preputial Gland — The preputial glands are one of the accessory sex glands of the male mouse composed of modified sebaceous acini and squamous ducts. Acute suppurative and chronic inflammation are common.

Preputial Gland Abscess — Abscesses of the preputial gland are frequently seen in male mice as a consequence of fighting. The abscess is characterized by numerous neutrophils and liquefactive necrosis.

Neoplastic Lesions

Neoplasms of Accessory Sex Glands — Neoplasms of all of the male accessory sex glands (prostate, seminal vesicles, preputial, and coagulating glands) are extremely rare. Adenocarcinomas of the prostate may be composed of relatively well-differentiated neoplastic cells. Squamous metaplasia may occasionally be seen. Adenomas and carcinomas of the seminal vesicle and coagulating gland have been reported very infrequently. Squamous cell papillomas and carcinomas are very occasionally seen in the preputial gland.

Integument

Nonneoplastic Lesions

Alopecia

Alopecia or hair loss on the face and back is common in mice and appears to be associated with friction contact with feeding jars. Alopecia also occurs on the thorax and abdomen of B6C3F1 mice, the cause of which is unknown (Burek et al., 1982).

Amyloidosis (Peckham, 1999)

Although amyloidosis of the skin is usually a manifestation of systemic amyloidosis, it has been infrequently observed in the skin of aging B6C3F1 mice primarily associated with the chronic inflammation due to mite infestation.

Atrophy

Atrophy of the skin is a sporadic spontaneous finding and is characterized by a very thin epidermis with associated atrophy of sebaceous glands and hair follicles. The causes of atrophy include nutritional deficiencies and hormonal imbalances such as hypothyroidism.

Ulcers

Ulcers are common spontaneous findings in the B6C3F1 mouse on NTP studies. Ulcers are deep excavations in the epidermis that penetrate into the underlying dermis. Ulceration is usually secondary to traumatic injuries or neoplasia. Ulceration secondary to an immune complex vasculitis was observed in groups of C57BL/6N mice (Andrews et al., 1994).

Dermatitis

Inflammation of the skin is a common finding in B6C3F1 mice. Fighting in group-housed animals and parasitism are predisposing causes.

Secondary inflammation is commonly associated with large sc neoplastic lesions as a consequence of impaired blood supply and trauma.

Neoplastic Lesions

General

Subcutaneous mesenchymal tumors are much more common in mice than are epithelial neoplasms (Goodman et al., 1981; Holland and Fry, 1982). Malignant mesenchymal tumors are usually named according to the predominant or most malignant pattern observed. However, in mice, these neoplasms appear to represent a spectrum. Because of this, some pathologists do not separate the different types and may group these tumors under a single term such as fibrosarcoma or sarcoma NOS (sarcoma not otherwise specified). These tumors are common in male B6C3F1 mice, and there is some evidence that they may be associated with fighting and chronic trauma in group-housed male mice (Squire, 1990).

Fibroma

These benign tumors are poorly to moderately cellular tumors (Ernst et al., 2001a). These tumors are composed of fusiform or stellate cells with elongated hyperchromatic or vesicular nuclei. The cells produce interlacing bundles of collagen fibers, which may be densely packed or loosely arranged if separated by edema or a mucinous ground substance. The tumors are relatively well circumscribed and noninvasive although they commonly cause local compression.

Fibrosarcoma

These malignant tumors are more cellular than their benign counterpart (fibromas) and produce less collagen. Pleomorphic spindle cells are arranged typically in either a herring bone pattern or forming interlacing bundles. Mitotic figures are numerous, and these tumors are locally invasive although they infrequently metastasize.

Fibrous Histiocytoma

These are the most commonly occurring soft tissue sc tumor in CD-1 mice. They are rare in B6C3F1 mice. Both benign and malignant histiocytomas occur although in CD-1 mice the latter are more common (Faccini et al., 1990b).

Benign Fibrous Histiocytoma

These tumors have a characteristic storiform pattern of neoplastic cells. The cell type is fibroblast like with a subpopulation of histiocytic cells. There is abundant collagen. Inflammatory cells are scattered throughout the tumor (Bruner et al., 2001).

Malignant Fibrous Histiocytoma

There are two main histological appearances of this malignant tumor of mesenchymal stem cells: fibrous and pleomorphic type. The fibrous type is composed of fibroblastic spindle cells arranged in a storiform pattern. Nuclei are pleomorphic, and mitotic figures are numerous. There is abundant collagen. Foci of necrosis and inflammatory cell infiltration are present.

The pleomorphic type is composed of rounded histiocytic cells with numerous bizarre multinucleated tumor giant cells and pleomorphic fibroblast-like cells. There are focal areas of collagen production.

Infiltration of surrounding tissue is a characteristic of both varieties of malignant fibrous histiocytoma although the pleomorphic type is more likely to metastasize.

Neurofibroma and Neurofibrosarcoma

These tumors arise from the fibroblasts of the perineural connective tissue. Their histological appearance is similar to fibromas and fibrosarcomas although association with a nerve is necessary for the diagnosis of neurofibromatous tumors.

Benign Schwannoma

These benign tumors arise from nerve sheath cells, the Schwann cell that originates from the neuroectoderm. There are two varieties of growth patterns in Schwann cell tumors, Antoni type A and Antoni type B. Antoni type A tissue is composed of relatively compacted cells that form whorls, bundles, and foci of nuclear palisading (Verocay bodies). Antoni type B tissue is composed of irregularly arranged cells in an edematous matrix.

Malignant Schwannoma

These malignant tumors are more commonly observed than the benign variety. Cell morphology is more pleomorphic. Local invasion and metastatic lesions are hallmarks of malignancy. Cells can be arranged in either Antoni type A or B patterns.

Sarcomas (Not Otherwise Specified, NOS)

Sarcoma NOS are extremely cellular tumors that may contain large bizarre nuclei, mitotic figures, and multinucleated giant cells. A pattern of interwoven bundles of fusiform cells may be apparent, but collagen fibers are difficult to demonstrate in any quantity even with polarized light. They may be locally invasive and metastasize.

Mast Cell Tumor

These neoplasms are typically benign and are usually solitary dermal nodules. Mast cell tumors are composed of densely packed polygonal mast cells with granular cytoplasm and a central round nucleus. There are few mitotic figures.

Malignant mast cell tumors are very uncommon and generally occur as systemic tumors.

Malignant Melanoma

These are primary tumors of the melanocytes that are of neuroectodermal origin. These tumors occur in the dermis. The neoplastic cells are spindle, epithelioid, or anaplastic. The neoplasm may be locally invasive. They are rare spontaneous tumors in mice that may be induced by topical application of carcinogens.

Epithelial Neoplasms

Epithelial tumors of the skin are rare spontaneous lesions in mice.

Basal Cell Adenoma

Basal cell tumors originate from primary epithelial germ cells of the piliary complex. Basal cell tumors have a variety of gross appearances.

Basal cell adenomas are circumscribed cellular masses with a variety of patterns and cell morphology. The cells are uniform, small, with prominent round or oval nuclei and relatively scant cytoplasm. Mitoses may be numerous. There is a variable fibrous stroma.

Basal Cell Carcinoma

These are distinguished from adenomas by abnormal or atypical cell morphologies, numerous mitoses, and frequently extensive local invasion.

Sebaceous Cell Adenomas and Carcinoma

Sebaceous cell adenomas are composed of sharply demarcated lobules of well-differentiated sebaceous cells. They may have foci of squamous epithelium with keratinization.

Sebaceous gland carcinomas are more irregular less organized masses than adenomas. The cells also tend to be less differentiated, and there may be local invasion.

Trichoepithelioma

These are a subtype of basal cell tumors and are tumors of the hair follicle.

They are generally sc and are characterized by the formation of small keratin cysts or hair shafts.

Basosquamous Tumor

A mixed piliary complex tumor containing both basal and squamous cells.

Keratoacanthoma

Benign neoplasms of basal and squamous epithelia that are crateriform or invaginated into the dermis and subcutis. They are thought to originate from hair follicles. Squamous cell carcinomas may arise from keratoacanthomas.

Squamous Cell Papilloma

These are superficial benign tumors arising from the epidermis and are composed of squamous epithelial cells that show acanthosis, hyperkeratosis, and papillary patterns. Chemical carcinogens readily induce squamous cell papillomas in mice during skin painting studies. These induced papillomas may become squamous cell carcinomas (Rehm et al., 1989).

Squamous Cell Carcinomas

These are malignant tumors of squamous epithelial cells that have a variable amount of keratin depending on the degree of maturation of the neoplastic cells. The tumors are composed of irregular masses or cords of squamous epithelial cells invading the adjacent dermis and subcutis.

Liver and Biliary System

Liver

Nonneoplastic Lesions

Extramedullary Hematopoeisis — Extramedullary hematopoeisis is normally present in both the fetal and neonatal mouse livers. The lesion may also occur in the adult mouse liver secondary to an infectious disease, hemorrhage, or neoplasia. When the predominant cell type is the granulocyte, granulocytic hyperplasia may be specified, and if nucleated erythrocytes are the prominent cells, erythropoietic hyperplasia is an appropriate term. In the mouse liver, the granulocytic activity seems maximal in the sinusoids adjoining the portal vein (Dunn, 1954).

Megakaryocytes may be associated with the areas of hematopoiesis.

Fatty Metamorphosis (Fatty Change, Steatosis, Lipidosis Fat Deposition) — Fatty metamorphosis may occur spontaneously in mice and also as a response to toxic agents (Harada et al., 1996, 1999). It may be present as a focal, zonal, or diffuse change. The degree of fatty metamorphosis may vary. It occurs spontaneously in 25%–30% of B6C3F1 mice at 24 months of age. The empty clear vacuoles with the peripherally located compressed nuclei represent lipid that has been removed during tissue processing. The lipid can be confirmed by staining frozen sections with Oil Red O or Sudan Black B. Fatty change is usually reversible, but sometimes when severe, it may result in both fibrosis and regenerative hyperplasia.

Glycogenic Vacuolation — Glycogenic vacuolation appears as irregularly shaped, clear vacuoles within hepatocytes. Glycogen is dissolved in the aqueous fixative. The glycogen content of the liver is variable depending on the physiological status of the mice (Faccini et al., 1990a). Overnight fasting causes a dramatic reduction in the amount of glycogen present in the liver. It is, therefore, important to randomize the time of sacrifice between treated and control animals.

Hemosiderosis — Hemosiderin pigment may be found in Kupffer's cells within the liver. The pigment is a distinctive granular, golden color within the cytoplasm of Kupffer's cells. Occasionally

special stains such as Prussian blue may be used to confirm the presence of iron and to differentiate it from other pigments such as bile or ceroid.

Ceroid Pigment (Lipofuscin) — Ceroid is a lipofuscin pigment that is acid fast and is acid Schiff (PAS) positive. An increase in ceroid pigmentation has been associated in aging mice in certain organs, including the ovaries and adrenals. Ceroid pigment in the liver may also occur as a result of hepatic toxicants. The pigment is slightly darker brown than hemosiderin and is usually present in the cytoplasm of Kupffer's cells adjacent to portal areas.

Necrosis — Focal hepatic necrosis is a nonspecific entity quite often encountered as an incidental finding in the liver of mice. It can be the result of viruses (mouse hepatitis virus), bacteria (*Bacillus piliformis*), toxicants, and ischemia; however, the etiology is often unknown. It may involve single cells, single or multiple lobules, and it may vary in distribution. Coagulation necrosis with distinct eosinophilic cytoplasm and pyknotic or absent nuclei is the typical morphological feature. Cell outlines are usually distinct, and the presence or type of an associated inflammatory reaction depends upon the duration of the lesion. Hepatic necrosis, which is induced by chemicals, may have a zonal distribution. If the extent of necrosis is severe and persistent, there may be a resultant fibrosis and compensatory hepatocellular hypertrophy.

Microgranulomas — Microgranulomas are commonly seen in small numbers in the livers of many strains of mice. They consist of small collections of macrophages and lymphocytes within the sinusoids or surrounding a single necrotic hepatocyte. The cause of these lesions is unknown. Periodic showering of bacteria from the intestine through the bloodstream has been suggested as a possible cause.

Inflammatory Lesions — Inflammatory lesions may occur as a result of a toxic agent. Inflammation may also result from infection with viruses and bacteria such as mouse hepatitis virus, mousepox, *Bacillus piliformis*, and *Helicobacter hepaticus.* In 1992, a novel chronic active hepatitis, which was associated with a high incidence of hepatocellular neoplasia, was identified in strain A/JCr control mice (Ward, 1984). *Helicobacter hepaticus* was found to colonize the gastrointestinal tract of many mouse strains and to demonstrate strain-dependent pathogenicity. Warthin Starry silver stains are used to demonstrate helical bacterial organisms. In mice of susceptible strains, the early liver changes are typically focal, acute, non-suppurative necrosis of hepatocytes. Later lesions are characterized by chronic active hepatitis with hepatocytomegaly, oval cell hyperplasia, cholangitis, and bile duct proliferation.

Karyomegaly and Cytomegaly — A striking histological feature of the livers of aged mice is the presence of hepatocytes with enlarged nuclei of variable size. The enlarged nuclei may be rounded or elongated and generally are two or more times normal in size. The polyploid cells appear with increasing frequency as aging occurs (Jones, 1967). This increase in nuclear size (karyomegaly) may or may not be associated with an increase in cell size (cytomegaly), and cytomegaly may occur either with or without an increase in the size of the nucleus. These changes have also been seen in mice treated with DDT, phenobarbital (Ward, 1984) Aroclor 1254 (Kimbrough and Linder, 1974), and other chemicals and in mouse hepatitis virus-infected cells (Ward et al., 1977). Toxins also often induce binucleated and multinucleated hepatocytes.

Inclusions — Both intranuclear and intracytoplasmic inclusions are frequently observed within normal and neoplastic mouse hepatocytes. Intranuclear inclusions are round, often filling most of the nucleus, and are distinctly eosinophilic in appearance. These inclusions have been reported to increase in incidence with age and are usually considered to be invaginations of the cytoplasm into the nucleus (Andrew, 1962; Herbst, 1976).

Cytoplasmic inclusions are somewhat less common and are most frequently seen in hepatocytes in or adjacent to hepatocellular neoplasms. Intracytoplasmic inclusions are round, vary markedly in size, and are usually eosinophilic. Some investigators have reported these intracytoplasmic inclusions to be aggregates of smooth endoplasmic reticulum (Hruban et al., 1966) or Mallory bodies. These types of intracytoplasmic inclusions have been reported in mice (Frith and Ward, 1980).

Atrophy — Atrophy that is diffuse in distribution may be related to administration of test substance or it may reflect inanition. The hepatocytes are small and appear more eosinophilic due to a decrease in the amount of glycogen.

Angiectasis — Angiectasis consists of widely dilated vascular spaces that are lined by unremarkable epithelial cells. This can range from one or two blood-filled cystic spaces to many.

Bile Duct and Ductular Hyperplasia — Proliferation of hepatic bile ducts may occur in response to toxicants and is sometimes associated with an inflammatory reaction. The lesion is usually diffuse, and many bile ducts are usually present in portal triads. Bile ductules (cholangioles, oval cells) may become hyperplastic in response to toxicants as well. Normal and hyperplastic bile ducts and ductules stain for keratin.

Oval cell hyperplasia proliferation of small, oval cells between hepatocytes frequently in periportal regions sometimes with the formation of incomplete duct-like structures. Oval cell hyperplasia is frequently associated with severe hepatocellular injury such as hepatocarcinogens and infection with *Helicobacter hepaticus.*

Cholangiofibrosis (Adenofibrosis) — Cholangiofibrosis is characterized by focal areas of basophilic atypical ducts in a fibrous stroma. It is rarely seen in control mice. The lesion is controversial and much less commonly induced in the mouse than in the rat. Adenofibrosis has been described in the mouse induced by Aroclor 1254 (Kimbrough and Linder, 1974).

Cirrhosis — Hepatic cirrhosis, whether postnecrotic, biliary, pericellular, or of some other type, is uncommon spontaneously in mice. It may be seen after a variety of toxicants, including carbon tetrachloride and chronic mouse hepatitis infection in nude mice (Ward et al., 1977). It often takes the form of a focal or diffuse increase in fine reticular fibers rather than distinct fibrous collagenous bands (Ward et al., 1979a).

Cholangitis — Cholangitis is inflammation of the bile ducts and is characterized by the presence of inflammatory cells (polymorphonuclear leukocytes and/or mononuclear cells) within the ducts and periductular tissue.

Hypertrophy — Hypertrophy of hepatocytes can be induced by a large number of compounds. Phenobarbital causes hepatocellular hypertrophy that is predominantly centrilobular and is due to smooth endoplasmic reticulum proliferation. Some hypolipidemic agents cause liver enlargement in mice. Typically, the hepatocytes are hypertrophied throughout the liver lobule and exhibit a ground-glass appearance of their cytoplasm. Electron microscopy in these cases reveals a marked increase in peroxisomes.

Hyperplasia, Hepatocellular Regeneration — This occurs as a consequence of liver damage and must be differentiated from a neoplastic lesion (Deschl et al., 2001). There is evidence of hepatocellular insult, for example, inflammation, fibrosis, necrosis, and atrophy.

Hepatocellular Foci of Cellular Alteration — Foci of cellular alteration in mice are somewhat similar to those described in the rat (Squire and Levitt, 1975) and may be seen in mice exposed to some carcinogens, including benzidine (Frith and Dooley, 1976), aldrin and dieldrin (Reuber, 1976), *N*-nitrosodimethylamine, *N*-nitrosodiethylamine (Ward, 1984; Ward et al., 1983), and other chemicals (Frith and Ward, 1980). They are occasionally seen in control mice. The incidence of hepatocellular foci is less than in rats. The primary alteration involves tinctorial qualities and textural appearances of the cytoplasm and size of hepatocytes. There is no obvious disruption of the liver architecture, and the affected hepatocytes merge with adjacent hepatocytes with little or no compression of adjacent normal parenchyma. The hepatic plates merge imperceptibly with the surrounding hepatocytes. Foci of cellular alteration may be classified as eosinophilic, basophilic, vacuolated, clear cell, or mixed. They may progress to adenomas and occasionally to carcinomas (Frith and Ward, 1980; Ward, 1984).

Clear cell foci consist of cells with a clear, ground-glass, or sometimes a lacy cytoplasm containing much glycogen. The clear areas stain with PAS stain prior to but not after diastase digestion, suggesting the presence of glycogen. The nuclei of the affected cells are not flattened against the cell membrane as in the vacuolated cells, but often are located in the center of the involved cells and surrounded by clear cytoplasm. Often, clear cell foci also contain many hepatocytes with basophilic cytoplasm.

Basophilic foci consist of hepatocytes that have increased basophilia of the cytoplasm. This basophilia is due to free ribosomes or rough endoplasmic reticulum. The cells are usually smaller than normal hepatocytes.

Eosinophilic foci are composed of slightly larger hepatocytes than normal with distinct granular pale pink cytoplasm.

Mixed-cell foci contain, in varying proportions, two or more of any of the cell types described in the preceding paragraphs.

Features that distinguish hepatocellular adenomas from foci of cellular alteration are cytomorphological features, growth pattern (i.e., loss of normal architecture), and compression of adjacent hepatic parenchyma.

Focal Fatty Change — Focal fatty change consists of focal areas of hepatocytes that contain lipid-laden, distinct cytoplasmic vacuoles of variable size. The nuclei of these vacuolated cells in microscopic sections are either absent or are flattened against the cytoplasmic membrane. Some pathologists use the term vacuolated cell foci of cellular alteration for this lesion.

Neoplastic Lesions

General — Hepatocellular neoplasms—both induced and spontaneous—are among the most common tumors in the mouse. The incidence of liver tumors in mice is very high in some strains, and the incidence can be affected by various factors. Consequently, the mouse liver tumor has been criticized as an inappropriate endpoint in carcinogenesis bioassays. There is probably no other tumor that has created more difficulty and confusion in the interpretation of its morphology, biology, and significance (Frith, 1979; Popp, 1984; Tomatis et al., 1973; Turusov and Takayama, 1979; Ward et al., 1979c).

The terminology used to describe liver tumors in the mouse has been varied and inconsistent. Up to now, some pathologists have considered a hepatocellular neoplasm to be a "true" tumor only if it metastasizes, whereas others diagnose all mouse liver neoplasms as hepatocellular carcinoma regardless of their morphology and biological behavior.

A variety of terms, including hyperplastic nodule, nodular hyperplasia, neoplastic nodule, hepatoma, liver cell tumor, liver cell adenoma, hepatocellular adenoma, liver cell carcinoma, and hepatocellular carcinoma have been used to describe neoplastic hepatocellular lesions of mice.

"Hepatocellular neoplasm" is a collective term used to describe the progressive stages of tumor development from the benign hepatocellular adenoma to the morphologically and biologically malignant hepatocellular carcinoma. Inasmuch as experimental pathologists, particularly those involved in carcinogenicity testing, need to distinguish between nontumors and tumors as well as between benign and malignant tumors, and since special biological and histochemical methods are either not helpful or simply cannot be used in large-scale studies, an attempt has been made to classify hepatocellular neoplasms on a purely morphological basis. The preferred term is hepatocellular adenoma for benign tumors and hepatocellular carcinomas for malignant tumors (Frith and Ward, 1980).

Hepatocellular Adenoma — The term "hepatocellular adenoma" is the preferred term for the morphologically and biologically benign liver cell neoplasm (Butler and Newberne, 1975; Gellatly, 1975; Reuber, 1971; Vesselinovitch and Mihailovich, 1983; Ward, 1984; Ward and Vlahakis, 1978). Adenomas are progressively growing focal lesions and may represent early stages in the formation of carcinomas (Frith and Ward, 1980; Ward and Vlahakis, 1978). Synonyms for this lesion include benign hepatoma, hyperplastic nodule, nodular hyperplasia (Butler and Newberne, 1975), type A nodule (Walker et al., 1972), type 1 or 2 nodule (Gellatly, 1975), liver tumor (Tomatis et al., 1972), neoplastic nodule (Squire and Levitt, 1975), and hepatocellular carcinoma. Hepatocellular adenomas are usually 1–10 mm in diameter, consist of cells resembling relatively normal hepatocytes, and usually contain cells similar to those in the foci of cellular alteration. Adenomas exist as distinct nodules that compress adjacent parenchyma and may bulge from the liver surface. Histologically, they are composed of a uniform population of well-differentiated cells that form a solid nodule and that may be composed of larger or smaller than normal hepatocytes that have cytoplasm that is basophilic, eosinophilic, or vacuolated. Adenomas may form regular plates one cell thick. They do not invade adjacent parenchyma or vessels, the lesions do not metastasize, and the small nodules have a lower degree of transplantability than do carcinomas (Gellatly, 1975; Reuber, 1967; Williams et al., 1979). Transplantability is the evidence of their neoplastic nature. More recently, they have been shown to be of clonal origin (Rabes et al., 1982), and some tumor cells contain alpha fetoprotein, which is also evidence of neoplasia (Koen et al., 1983). Some chemicals induce either eosinophilic (Hoover et al., 1980; Ward, 1984; Ward et al., 1979c) or basophilic adenomas (Vesselinovitch et al., 1978; Ward et al., 1979b). Naturally occurring hepatocellular carcinomas can arise within adenomas (Frith and Dooley, 1976; Ward, 1984; Ward and Vlahakis, 1978).

These foci of carcinoma within adenomas appear identical to carcinomas described in the following.

Hepatocellular Carcinoma — The diagnosis of hepatocellular carcinoma is often made on a distinct trabecular or adenoid pattern as well as on cytological features characteristic of malignancy. Synonyms for this lesion include type B nodule (Walker et al., 1972), type 3 nodule (Gellatly, 1975), trabecular carcinoma, and malignant hepatoma. The liver cell plates are three or more cell layers thick, irregular, and composed of well to poorly differentiated hepatocytes. The well-differentiated tumors are composed of uniform cells with a fair amount of cytoplasm. The moderately well-differentiated hepatocellular carcinomas are composed of larger hepatocytes, which vary more in size and shape and form thickened trabeculae or a solid pattern. The poorly differentiated tumors are composed of cells with less cytoplasm and more immature nuclei forming prominent plates or a solid pattern; some have extremely large anaplastic cells.

The incidence of metastases has generally been considered very low for mouse hepatocellular tumors, but more recent studies indicate that a thorough examination of step or serial sections of the lung reveals a much higher incidence than previously expected, especially for tumors induced by dimethylnitrosamine (Kyriazis et al., 1974). Metastases are usually uncommon (0%–5%) in mice

with spontaneous hepatocellular carcinomas (Butler and Newberne, 1975), but they may be seen in up to 40% of male B6C3F1 or C3H mice with hepatocellular carcinoma that are allowed to live out their life span. Metastases usually occur only when tumors are large (over 10 mm) and increased in weight (Frith and Ward, 1980). Some chemicals cause highly metastatic tumors, whereas other cause carcinomas with a low metastatic rate (Ward, 1984). Pulmonary metastases may share the trabecular pattern often seen in the primary hepatocellular carcinomas, or they may be more solid in appearance.

Hepatoblastoma — Hepatoblastomas have been described in mice as occurring spontaneously and are also experimentally induced. Turusov et al. (Turusov and Takayama, 1979) described liver tumors in mice that resembled human hepatoblastomas. These tumors are almost invariably found within or adjacent to hepatocellular carcinomas and are readily distinguished by their basophilia with H&E stain. The tumors frequently consist of organoid structures lined by vascular channels. The channels are surrounded by several layers of tumor cells arranged either radially or concentrically. In some areas, the cells are arranged in rows, rosettes, sheets, or ribbons. Foci of osseous metaplasia are occasionally seen. The cell of origin for this particular mouse neoplasm is uncertain, although it has been suggested that this type is of fetal origin. While similar neoplasms occur in children, hepatoblastomas occur only in aged mice. Other pathologists have referred to similar lesions as poorly differentiated cholangiocarcinomas (Reuber, 1967) and cholangiomas (Jones, 1967; Vlahakis and Heston, 1971). Recently, the presence of keratin in hepatoblastomas has been demonstrated in aged mice but not alpha fetoprotein, suggesting their duct or ductular origin rather than their hepatocellular origin.

Cholangioma and Cholangiocarcinoma — Cholangiomas and cholangiocarcinomas are relatively rare in mice compared to the common occurrence of hepatocellular neoplasms (Reuber, 1967; Vlahakis and Heston, 1971). Some hepatocellular carcinomas assume a distinctly adenoid or glandular pattern suggestive of an adenocarcinoma, yet in some cases, when the transition is not evident, it is sometimes difficult to differentiate a hepatocellular adenocarcinoma from a cholangiocarcinoma. Mixed carcinomas, or carcinomas containing distinct areas of both trabecular hepatocellular carcinoma and cholangiocarcinoma, have also been described.

Metastatic Tumors — Metastatic tumors of the livers are rare compared to metastatic tumors of the lung. The most common are the hematopoietic neoplasms, including lymphoblastic lymphoma and FCC lymphoma. Histiocytic sarcoma may occur as either a metastatic or a primary neoplasm of the liver. Metastatic tumors in the liver include alveolar/bronchiolar adenocarcinoma, urinary bladder transitional cell carcinoma, osteosarcoma, and pancreatic islet cell carcinoma (Frith, 1983).

Gallbladder

Nonneoplastic Lesions

Gall Stones — Gall stones are rare in mice and have not been chemically characterized. Stones are present in the lumen and consist of concentric laminations or lamellae.

Crystals — Distinct eosinophilic crystals of obscure etiology are occasionally seen in the cytoplasm of the epithelial cells and in the lumen of the gallbladder of mice. They may be similar in etiology to those described in the lungs, and may be products of the epithelium, since the acidophilic cytoplasm of the epithelial cells stains and reacts the same as the extracellular crystals.

Neoplastic Lesions

Adenoma — Gallbladder adenomas are rare tumors in the mouse. They consist of papillary projections covering a connective tissue core.

Adenocarcinoma — A single gallbladder carcinoma has been seen in a control mouse at NCTR, and a small number have been seen in the B6C3F1 mouse (Yoshida et al., 1986). These tumors may be broad-based masses or diffuse thickening of the mucosa.

Nervous System

Nonneoplastic Lesions

Cerebral Mineralization

Mineralized deposits detected in the cerebrum generally occur in the region of the medial, ventral, and posterior thalamic nuclei (Morgan et al., 1983). These deposits are very common. However, the incidence is quite variable and can depend upon the level of the brain from which the sections were taken. They vary from single, rounded bodies up to 100 mm in. diameter to very extensive, irregular, but usually rounded masses, which in most cases are found to be associated with blood vessels. They appear to develop on vascular basement membranes, and focal malacia of the neuropil can result. Focal osseous metaplasia can occur in the neuropil but is more common in the meninges (Radovsky and Mahler, 1999).

Hydrocephalus

Hydrocephalus may be congenital or more commonly is associated with obstructed outflow of CSF, for example, secondary to compression by an underlying pituitary tumor (Radovsky and Mahler, 1999). Hydrocephalus usually involves the lateral ventricles, is bilateral, and is characterized by increased fluid in the ventricular system. The ventricles may be so enlarged as to cause a dome shape to the cranium. The sulci and gyri may be flattened and almost indiscernible in advanced cases. Microscopically, the ventricles are enlarged at the expense of the flattened cerebral cortex.

Vacuolization of the White Matter: Central Nervous System

Spongiosis and vacuolization refer to microscopic cystic degeneration of gray or white matter. Vacuoles are a common entity in the white matter of the brains of mice, particularly in the cerebellum. Vacuoles may be present within neuronal cytoplasm, with or without neuronal dropout and reactive astrogliosis, or caused by distension of myelin sheaths indicative of myelin edema or demyelination (Radovsky and Mahler, 1999). It has been suggested that vacuolization may be an artifact associated with the collection and processing of the brain (Wells and Wells, 1989). Pathological vacuolization can be difficult to differentiate from artifactual change, but typically vacuoles have less regular outlines and may contain cellular debris (Radovsky and Mahler, 1999).

Infarct

Infarcts are rare in the central nervous system, but occasionally occur in the cerebrum. They more commonly occur at the surface or less frequently deeper within the parenchyma.

Developmental Abnormalities

Epithelial inclusion cysts are a relatively common developmental abnormality of the brain and spinal cord, and there may be considerable variation in size. Cysts are lined by flattened squamous epithelium and are filled with eosinophilic desquamated keratin. There are seldom any associated clinical signs.

Adipose tissue accumulations ("lipomas") have been seen associated with both the meninges and choroid plexus (Morgan et al., 1984). They consist principally of discrete, well-demarcated clusters or masses of mature adipocytes. They may be present in the interstitium of the choroid plexus of the lateral ventricles. Occasionally, a small mass of cartilage-like material is present among the fat cells. The cell or tissue of origin of these lesions is unknown. Others consider these lesions to be a developmental abnormality (Radovsky and Mahler, 1999).

Neoplastic Lesions

A number of cell-specific markers for the murine nervous system are useful in the differentiation of tumors and include neurofilament markers for neurons; glial fibrillary acidic protein (GFAP) for glial cells and myelin basic protein (MBP) for oligodendrocytes and Schwann cells. NSE and S-100 protein are less specific markers for neural tissue.

Oligodendroglioma — Tumors of the central nervous system of the mouse are extremely rare lesions (Morgan et al., 1984). The most common tumor is the oligodendroglioma that occurs in the cerebrum and/or diencephalon. It is usually ventrolateral in location and involves much of the thalamus, hypothalamus, and amygdaloid. The oligodendroglioma comprises a poorly demarcated mass of proliferating oligodendrocytes with variable degrees of neuronal satellitosis, nuclear palisading, and mitoses. These cells have scanty cytoplasm, with a distinct perinuclear halo in some cases; the single round-to-oval nuclei are often hyperchromatic.

With immunohistochemical staining, they are positive for MBP.

Astrocytoma — Astrocytomas occur much less frequently than oligodendrogliomas in mice. The neoplasms comprise cells with indistinct cytoplasmic boundaries and large, oval, or slightly folded nuclei. The margins of the tumor mass are indistinct, and there may be areas of edema, hemorrhage, and microcystic damage. Hemorrhage and necrosis are seen more frequently in astrocytomas than in oligodendrogliomas.

Differential diagnoses should include benign mixed glioma and reactive gliosis (Krinke et al., 2001a).

Malignant astrocytoma extends over multiple areas in the CNS and may be multicentric. Invasive growth into perivascular spaces and meninges is characteristic of these neoplasms. There is a high degree of cellular atypia, pleomorphism, necrosis, and hemorrhage.

Astrocytomas in mice, unlike those in man, are commonly GFAP negative, despite their fibrillary differentiation.

Meningioma — Meningiomas are typically discrete, expansile masses overlying the brain or spinal cord. They occur rarely in the mouse and can assume a variety of patterns as in other species. These neoplasms comprise a regular pattern of loosely interwoven bundles of delicate spindle cells, with single small hyperchromatic oval nuclei. The nuclei in some areas palisade in an irregular fashion. These cells lie in a faintly basophilic finely granular ground substance. In some cases, the neoplasms infiltrate the ventral brain along the adventitia of small blood vessels. Malignant meningioma is diagnosed when there is evidence of local invasion.

Major differential diagnoses are metastatic sarcoma and systemic histiocytic sarcoma (Radovsky and Mahler, 1999).

Metastatic Tumors — Leukemias and lymphomas commonly involve the brain. Mesenchymal tumors (sarcomas) may also occasionally spread to the brain. Carcinoma of the pituitary gland and nasal cavity may extend locally to involve adjacent brain.

Respiratory System

Upper Respiratory Tract (Nasal Cavity, Larynx, Trachea)

Spontaneous nonneoplastic and neoplastic lesions of the nasal cavity, larynx, and trachea are uncommon in mice.

Lung

Nonneoplastic Lesions

Alveolar Epithelial Hyperplasia — Alveolar epithelial hyperplasia is characterized by a single layer of nonciliated cuboidal cells lining all or part of the alveolar wall of the lung. The cells are found on the scaffold of existing stroma. There are neither papillary projections into the lumens nor solid masses of cells filling alveolar spaces. The proliferating cells are type II pneumocytes (Goodman et al., 1981). These lesions can be precursors of alveolar/bronchiolar neoplasms.

Preneoplastic hyperplasia is not associated with inflammation and necrosis, and the parenchymal architecture is usually maintained, which differentiates this lesion from regenerative hyperplasia (Dixon et al., 1999). Regenerative hyperplasia of the alveolar type II pneumocytes or airway epithelium commonly occurs in response to cell loss due to injury or cell death.

Alveolar Hemorrhage — Acute alveolar hemorrhage is a common agonal finding in mice (Percy and Barthold, 2001). It is differentiated from older hemorrhage or chronic heart failure by the absence of pigment (hemosiderin)-laden macrophages in the lumina.

Pulmonary Histiocytosis — Focal accumulations of lipid-laden macrophages (some of which may contain cholesterol lefts) are frequently observed in the subpleural areas of aging mice of all types (Percy and Barthold, 2001).

Alveolar Lipoproteinosis — Alveolar lipoproteinosis is characterized by intra-alveolar accumulations of vacuolated macrophages (pulmonary phospholipidosis).

Crystal Pneumonitis — Crystal pneumonitis is characterized by variably sized crystals, within macrophages or free in lumina of terminal airways, alveolar ducts, alveolar lumina, and bronchiolar glands in certain strains of mice, particularly aging B6 mice (Percy and Barthold, 2001). Immunodeficiency is associated with accelerated lesion formation. Crystals are composed of breakdown products of granulocytes, particularly eosinophils (Murray and Luz, 1990). Lesions may be extensive enough to cause dyspnea.

Inflammatory Lesions of the Lung — Differentiation of lesions associated with underlying infectious disease from those of toxic origin is obviously of critical importance in the interpretation of toxicological bioassays. The presence of infectious agents may also influence the toxic and/or neoplastic outcome of chemical administration (Dixon et al., 1999). The lesions associated with the principal infectious agents of rodent respiratory tract are outlined in the following.

Sendai Virus — Sendai virus is an extremely contagious pulmonary disease of mice caused by an RNA virus (Paramyxovirus). Its prevalence is considered to be high worldwide. Grossly, the lungs

are plum colored, atelectatic, and exude a serosanguinous fluid on cut surface. Microscopically, the lesions consist of necrotizing bronchitis and bronchiolitis with the formation of polyploid proliferations and proliferative alveolitis.

Mycoplasma pulmonis (Murine Respiratory Mycoplasmosis) — *Mycoplasma pulmonis* is a gram-negative bacterium that preferentially colonizes the luminal surface of respiratory epithelia. It has been also incriminated in natural infections of the genital tract in rodents. Lung lesions are characterized by a purulent bronchopneumonia with bronchiectasis.

Corynebacterium kutscheri — *C. kutscheri* is a gram-positive bacterium causing septicemia with bacterial emboli in the liver, kidney, lungs, skin, and joints.

Cilia-Associated Respiratory Bacillus (CAR Bacillus) — CAR bacillus is a gram-negative bacterium causing respiratory disease similar to severe murine respiratory mycoplasmosis.

Pneumocystis carinii — This is an opportunistic organism affecting immunosuppressed or immunocompromised animals. Histologically, alveoli are filled with eosinophilic foamy material, septae are usually thickened, and there are few inflammatory cells.

Lymphocytic Infiltrates — Lymphoid tissue is normally present in association with the bronchi. Lymphocytic infiltrates are very common around blood vessels and bronchioles or in the pleura as focal lesions. These lesions are usually minimal to mild in severity and are not diagnosed by some pathologists. They probably represent a response to antigenic stimuli, such as previous viral infection.

Such infiltrates are also present as part of a generalized perivascular change in lymphoproliferative disorders.

Neoplastic Lesions

General — The most common lung tumors observed in B6C3F1 and CD-1 mice are alveolar/bronchiolar adenomas and carcinomas. Many of these arise from type two pneumocytes. Tumors arising from Clara cell have also been described (Kauffman and Sato, 1985a,b; Theiss and Shimkin, 1982). On H&E sections, these two tumor types can be difficult to differentiate morphologically. Consequently, in most toxicology studies, the terms alveolar/bronchiolar (A/B) adenoma and carcinoma are used routinely. Squamous cell carcinoma of the lung can be induced by various agents, but are extremely uncommon spontaneous tumors.

Alveolar/Bronchiolar Adenoma — Alveolar/bronchiolar adenomas microscopically may be circumscribed and compress adjacent tissue, or the edges may be irregular where the neoplastic cells extend into adjacent airways. The lesion is composed of cells with a normal nuclear/cytoplasmic ratio that are cuboidal or columnar and resemble normal lining cells of the lower airway. Proliferating cells may form papillary projections into alveoli or completely fill them. The central portions of these tumors tend to be more solid than the periphery.

Alveolar/Bronchiolar Carcinoma — Alveolar/bronchiolar carcinomas tend to be larger than adenomas. There is cellular and nuclear pleomorphism, increased nuclear/cytoplasmic ratio, hyperchromatic nuclei, and an increase in mitotic figures. There may be multiple solid areas within the tumor, invasion of airways, blood vessels, lymphatics, and pleural surfaces; extension throughout the mediastinum; and coelomic and distant metastases. Necrosis is common: when these tumors metastasize, they may have a sarcomatous pattern.

Metastatic Tumors — The lung is the most common site of metastasis of neoplasms in rodents (Dixon et al., 1999).

Mammary gland neoplasms and systemic hematopoietic tumors are the two most frequently observed metastatic tumors (Sass and Liebelt, 1985; Vaage, 1988). Metastatic mammary gland neoplasms must be differentiated from alveolar/bronchiolar adenocarcinoma of the lung.

Special Senses

Eye

Nonneoplastic Lesions

Nonneoplastic lesions of the eye are occasionally observed in aged CD-1 and B6C3F1 mice. Most commonly observed, although infrequent and sporadic in incidence, are cataracts and retinal atrophy.

Retinal Atrophy — Age-related retinal atrophy in the mouse may be unilateral or bilateral and is characterized by loss of the photoreceptors (predominantly rods) and outer nuclear layers. The lesion may progress to loss of the second or third layer of neurons eventually resulting in a thin fibrous layer containing glial cells and occasional neurons (Geiss and Yoshitomi, 1999). Inherited retinal degeneration has been described in mutant and transgenic mice (Gregory and Bird, 1995; Sidman and Green, 1995; Smith, 1992).

The rodent retina, particularly that of the albino strains, is sensitive to increased light intensity (Greenman et al., 1982). Due to its pigmentation, the incidence in the B6C3F1 mouse is less than that in the albino BALB/c mouse (LaVail et al., 1987). Such light-induced changes are morphologically difficult to differentiate from early age-related changes.

Cataract — Cataract is occasionally seen in the lens of the eyes of mice. Morphologically, cataracts appear as multiple globoid circumscribed bodies within the substance of the lens, especially at the periphery or capsular surface. Histologically, all degenerative lesions of the lens capsule, epithelium, or fibers resulting in reduced light permeability or opacity are termed cataracts (Geiss and Yoshitomi, 1999).

Early lenticular degeneration consists of irregular swelling of the epithelium or fiber cells with a granular or vacuolated cytoplasm, progressing to liquefaction and Morgagnian globule formation, and, often, dystrophic mineralization.

Senile cataract formation is associated with decreased superoxide dismutase activity and increased free radical–induced damage.

Accumulations of polyol and glycolation of protein are important in the pathogenesis of cataract formation in diabetic mice.

Neoplastic Lesions

Primary tumors of the eye are extremely rare in all mouse strains (Squire et al., 1978).

Secondary tumors such as malignant lymphoma and Harderian gland carcinoma can spread to the eye (Geiss and Yoshitomi, 1999).

Ear

Vestibular Syndrome

Signs of vestibular disease such as head tilt and circling are common in mice. Causes include bacterial otitis, CNS disease, and necrotizing arteritis. The latter is characterized by segmental necrosis and/or inflammation of medium-sized arteries in tissue surrounding the structurally normal internal and middle ear.

The etiology of the arteritis is unknown, but in some strains, it may be immune mediated (Andrews et al., 1994; Plendl et al., 1996).

Harderian Gland

Nonneoplastic Lesions

Small focal lymphocytic infiltrates and ectasia of the ducts are occasionally observed.

Neoplastic Lesions

Harderian Gland Adenoma — These tumors are frequently well-demarcated lesions that cause compression of the adjacent parenchyma, but usually do not have a well-defined capsule. The architecture consists of pseudoglandular structures from which arborizing and folded fingerlike fronds project into the lumen. The fronds are composed of a delicate fibrovascular core covered by cells that are usually tall columnar with foamy amphophilic cytoplasm. The cells usually form a single layer, but areas are frequently present that give the appearance of having a basal layer of normal-appearing cells "capped" by smaller cells adjacent to the lumen.

Harderian Gland Adenocarcinoma — These tumors maybe well differentiated, differing from adenomas by greater cellular atypia, invasion, and metastases. Undifferentiated adenocarcinomas contain areas of varying size that are solid and composed of pleomorphic cells, many of which contain a single large vacuole. Mitotic figures are uncommon.

Urinary System

Kidney

General

Mouse Urinary Protein — Mouse urinary protein is a protein similar to α 2u-globulin produced by male rats, but unlike α 2u-globulin, it is not reabsorbed by the kidney. Hyaline droplet nephropathy therefore does not occur in the mouse (Lehman-McKeeman and Caudill, 1992).

Sexual Dimorphism of the Kidney — The glomerular tuft of the adult male mouse kidney of certain strains has a unique morphological characteristic that is not a pathological lesion. In females, the parietal layer of Bowman's capsule is composed of a single layer of flattened epithelium. In the male mouse, the parietal layer of most of the glomeruli is composed of simple cuboidal epithelium. It is not present in each glomerulus, and the proportion of glomeruli showing this characteristic varies in different strains. The presence of cuboidal epithelium is thought to be due to metaplasia of flattened cells under the influence of testosterone (Leibelt, 1986).

This characteristic is seen only in adult male mice possibly associated with their greater requirement for reabsorption of albumin and prealbumin. Proteinuria, due to the high albumin and prealbumin content, is normally seen in mice but is increased in mature males because of the influence of testosterone (Percy and Barthold, 1993).

Congenital Lesions — Congenital lesions of the kidney vary between strains. Agenesis, fusion, hypoplasia, and displacements have been reported. Polycystic renal disease has been reported in several mouse strains.

Double Renal Pelvis — The kidney of the mouse contains a single large renal papilla surrounded by the renal pelvis. Very rarely, a congenital anomaly consisting of a double renal pelvis may occur.

Hydronephrosis — Hydronephrosis is a fluid-induced dilation of the renal pelvis, which may be minimal, resulting in only a slight dilatation of the renal pelvis, to severe causing marked compression atrophy of the kidney. It may be unilateral or bilateral, congenital or acquired, and frequently results in tubular dilatation. A high incidence of congenital hydronephrosis occurs in the C3H, C57L, and DDD strains (Hsu, 1986). Progressive renal failure has been reported in association with congenital hydronephrosis in C57BL/6 mice (Horton et al., 1988).

   Acquired hydronephrosis may follow obstruction of one or both ureters or the urethra due to calculi, tumors, or inflammation (Bendele and Carlton, 1986; Carlton and Gries, 1983). Papillary necrosis secondary to amyloidosis can also result in hydronephrosis.

Degenerative Lesions

Nephropathy — Chronic nephropathy similar to that seen in rats also occurs in mice (Montgomery, 1986; Wolf and Hard, 1996), but lesions tend to be less severe. The condition can occur spontaneously or be drug induced (Wolf, 1996, #56).

   The incidence and severity of lesions is greater in males than in females. Microscopic changes include focal to multifocal tubular basophilia and nuclear crowding. Tubular basement membranes may be thickened, and in more advanced lesions, eosinophilic proteinaceous casts are present within the lumina (Wolf, 1996, #56). The interstitium may contain variable mononuclear cell infiltrates. Glomerular changes progress from hypercellularity of the glomerular tuft and dilatation of Bowman's space to glomerulosclerosis.

Hyaline Glomerulonephropathy — Eosinophilic hyalinization of glomeruli associated with PAS-positive subendothelial deposits resembling immune-mediated glomerulonephritis has been reported in B6C3F1 mice (Wojcinski et al., 1991).

Renal Papillary Necrosis — Necrosis of the renal papilla may occur in the mouse kidney and is usually associated with renal amyloidosis (Frith and Ward, 1988) or toxins. Papillary necrosis may be seen secondary to ascending pyelonephritis such as that occurs following bite wounds in group-housed males.

Renal Mineralization — The degree of renal mineralization varies between strains and does not appear to have a sex predisposition in mice as it does in rats. Mineralization is seen microscopically as densely basophilic material associated with tubular basement membranes or blood vessels, particularly in the corticomedullary junction. Intraluminal mineral may also be seen.

   Renal calculi may occur within the renal pelvis (Frith and Ward, 1988).

Renal Infarct — Renal infarcts in the mouse are morphologically comparable to renal infarcts in other species. They commonly appear as triangular-shaped areas of coagulation necrosis, which extend from the capsular surface through the cortex to or into the medulla. The tubules may be intensely eosinophilic, and fibrosis may be present in older lesions. The surface of the infarct is depressed below the normal renal surface. In recent lesions, polymorphonuclear leukocytes may infiltrate the border, and some tubules may contain hemoglobin casts. Infarction may occur secondary to arteriothrombosis, disseminated intravascular coagulation, pyelonephritis, amyloidosis, or neoplasia (Seely, 1999).

Neoplastic Lesions

The most common renal proliferative changes are tubular epithelial hyperplasia, adenoma, and carcinoma. Renal cell adenoma and carcinoma may occur spontaneously (Shinohara and Frith, 1980) or can be induced.

A progression of change from hyperplasia through adenoma to carcinoma can be seen with the administration of renal carcinogens but is not inevitable (Shinohara and Frith, 1980).

Renal sarcomas have been induced in mice by polyoma virus infection (Frith et al., 1994).

Renal Tubular Hyperplasia — Renal tubular hyperplasia has been interpreted as a precursor of neoplasia on several occasions (Shinohara and Frith, 1980; Terracini et al., 1966; Terracini and Testa, 1970). Single or multiple foci of change involving a single tubule may be seen. There is a lack of compression of surrounding parenchyma, and lesions are less than three times the diameter of a normal tubule. Hyperplastic epithelial cells, with a normal to minimally pleomorphic appearance, partially to completely fill the lumen. Occasionally, lumina may be dilated (Seely, 1999).

Tubular hyperplasia should be differentiated from tubular epithelial hypertrophy, consisting of normal numbers of enlarged, typically eosinophilic tubular epithelial cells, and from adenoma.

Tubular Cell Adenoma — Most renal tubular cell adenomas are single lesions that are sharply defined, often with some compression of the surrounding parenchyma and greater than five tubules in diameter. They can be morphologically classified as cystic, papillary, or solid (Shinohara and Frith, 1980). The papillary type is the most common. Several growth patterns can coexist within the same tumor.

The cells forming the adenomas are cuboidal with eosinophilic, basophilic, or clear cytoplasm and relatively small nuclei. There is minimal pleomorphism with some variation in cell size. Mitotic figures are rare.

Renal Tubular Cell Carcinoma — Renal tubular cell carcinomas tend to be larger than adenomas, with marked compression or infiltration of surrounding parenchyma. There may be marked hemorrhage and necrosis. Histologically, they can be solid, cystic, papillary, mixed, or anaplastic in appearance. Component epithelial cells may have eosinophilic, basophilic, or clear cytoplasm, and vary from small and uniform to large and pleomorphic. The nuclei are small and round or oval to highly pleomorphic with prominent nucleoli. The mitotic index in the tubular cell carcinomas varies from tumor to tumor (Seely, 1999). Some of these tumors contain areas of sarcomatous change with multinucleated giant cells (Frith et al., 1994). Carcinomas occasionally metastasize to the lungs.

Nephroblastoma — Nephroblastomas are rare tumors of undifferentiated (blastema) cells that may contain primitive tubular and glomerular structures (Leibelt et al., 1989; Turusov, 1992).

Neoplastic Associated Changes — Eosinophilic, hyaline droplets consisting of lysozyme secreted by monocytes and macrophages (Hard and Snowden, 1991) are found within renal tubules in association with histiocytic sarcoma of the kidney (Luz and Murray, 1991).

Renal Toxicity — The kidneys are highly susceptible to toxic injury by virtue of their large blood supply, their functions of filtration, concentration and excretion of xenobiotics, and metabolic capability resulting in tubular enzymatic bioactivation of some xenobiotics (Stine and Brown, 1996).

Renal tubular toxic changes are most readily recognized morphologically and comprise necrosis and/or degeneration and regeneration, particularly of the proximal convoluted tubule (Seely, 1999).

Degeneration is characterized by increased cytoplasmic eosinophilia and/or vacuolation. In tubular necrosis, there are intense cytoplasmic eosinophilia, nuclear pyknosis, or karyorrhexis, and epithelial desquamation into tubular lumina resulting in eosinophilic tubular casts. Regenerative changes comprise increased tubular basophilia, with nuclear crowding. Cytomegaly, karyomegaly, and dysplasia have also been reported (Abdo et al., 1984; Nakanishi et al., 1982). Increased mineralization in female mice has been reported with administration of 11-aminoundecanoic acid (Dunnick et al., 1983).

Papillary necrosis has been reported in association with chemical administration in mice (Wolf et al., 1992).

Ureter

Nonneoplastic

Ureteral Diverticulum — Diverticula are not common spontaneous lesions in the mouse but have been associated with inflammation and the administration of 4-ethylsulfonyl naphthalene-l-sulfonamide (ENS), acetazolamide, and oxamide (Frith et al., 1984; Jackson et al., 1979). The lesion exists as a downgrowth into the ureteral wall that may be confused with a carcinoma. Downgrowths of the surface epithelium may extend through the muscularis to the adventitia. The lesion appears to be associated with crystalluria.

Urinary Bladder

Nonneoplastic Lesions

Bladder Calculi — Urinary calculi may occur spontaneously or may be associated with the administration of compounds such as ENS (Frith et al., 1984; Jackson et al., 1979). Calculi are usually accompanied by chronic cystitis and may result in papillary and nodular urothelial hyperplasia.

Lymphoid Aggregates — Focal lymphoid aggregates may occur in the lamina propria of the urinary bladder. Their incidence and severity increase with age.

Urothelial Hyperplasia — Hyperplasia of the transitional epithelium of the mouse urinary bladder is a rare spontaneous lesion, but a common finding in association with chronic cystitis, calculi formation, and toxic compounds such as cyclophosphamide and methyl or ethyl methanesulfonate.

Hyperplasia is also seen in animals treated with bladder carcinogens and may be considered a preneoplastic lesion with the potential to progress to a tumor (Koss, 1977). Hyperplasia may be focal, multifocal, diffuse, simple, papillary, or nodular (Frith, 1979).

Papillary hyperplasia is associated with thickening and folding of subjacent lamina propria and tends to be multifocal to diffuse, differentiating this lesion from the usually solitary transitional cell papilloma (Gaillard, 1999).

Nodular hyperplasia (transitional epithelial downgrowths extending into the lamina propria) may occur in conjunction with either simple or papillary hyperplasia and may be focal or multifocal. Nodular hyperplasia is comparable morphologically to von Brunn's nests or cystitis cystica in humans (Frith, 1979). Although the areas of nodular hyperplasia often appear to have no connection with the surface epithelium, serial sections usually reveal such a connection. The lesion may regress if the etiological stimulus is removed (Frith and Rule, 1978).

Urothelial Metaplasia — Urothelial transitional epithelium may undergo metaplasia to squamous or rarely glandular epithelium, in association with inflammation, hyperplasia, or neoplasia (Greaves, 1998; Roe, 1964).

Neoplastic Lesions

Urinary Bladder Papilloma — Naturally occurring papillomas of the urinary bladder are rare in mice. The epithelium shows no pleomorphism, atypia, or anaplasia, and is well differentiated. Papillomas induced with 2-acetylaminofluorene (2-AAF) may have a slender narrow stalk or a broad base (Frith, 1979).

Urinary Bladder Carcinoma — Naturally occurring bladder carcinomas are extremely rare in mice (Frith et al., 1980). Experimentally induced malignant neoplasms of the urothelium may be classified according to histological pattern, cell type, and depth of invasion. Malignant epithelial lesions classified by histological pattern may be divided into papillary and nonpapillary (solid, polyploid) carcinomas. Papillary carcinomas project into the lumen and may or may not show invasion. Nonpapillary or solid carcinomas invade the bladder wall.

   Bladder carcinomas classified according to histological cell type are divided into transitional cell, transitional cell with squamous metaplasia, squamous, undifferentiated, and adenocarcinoma types. The most common type induced is the transitional cell carcinoma.

Musculoskeletal System

Bone

Nonneoplastic Lesions

Fibro-Osseous Lesion, Bone — This lesion has been called by a variety of terms: fibro-osseous lesion, myelofibrosis, senile osteodystrophy, or osteoporosis (Burek et al., 1982; Frith and Ward, 1988; Goodman et al., 1981; Sass and Montali, 1980). This lesion is seen in the sternum and other bones in aging mice, particularly in females. There is replacement of all or part of the marrow cavity with an eosinophilic fibrillar matrix containing abundant collagen fibers. Fibroblast-like cells and osteoclasts are present throughout the matrix. Small foci of normal marrow may be present. Although the lesion resembles fibrous osteodystrophy associated with renal disease, the pathogenesis appears to be different as both kidneys and parathyroids are normal. Similar lesions have been induced with estrogen administration.

Ankylosis — Ankylosis of the hock joints has been reported in group-caged male B6C3FI mice (Rao and Lindsey, 1988). The incidence of ankylosis increases rapidly after 6 months of age. The cause is unknown, but the incidence decreased when mice were individually caged. The joints are enlarged. Microscopically, there are bony proliferations (exostoses) on the tarsal bones. There is bridging of the joint and, in severe cases, obliteration of the joint with new bone.

   Chronic exposure of CD-1 mice to sodium fluoride is associated with ankylosis of the stifle joint (Maurer et al., 1993).

Bone Atrophy — Bone atrophy is the proportional loss of mineralized and unmineralized matrix resulting from decreased formation and/or increased resorption of bone.

   It can occur as a spontaneous age-related lesion, predominantly in females.

   Localized bone loss may occur secondary to immobilization (Long and Leininger, 1999).

   Senescence-accelerated mouse strains have accelerated bone loss and associated spontaneous fractures (Takahashi et al., 1994).

Other Nonneoplastic Lesions — Osteoarthrosis and bone necrosis with associated fractures have also been reported sporadically (Burek et al., 1982).

Neoplastic Lesions

Osteomas and osteosarcomas are occasionally observed in mice.

Oncogenic tumor virus-induced osteomas can occur spontaneously in a number of different strains of mice (Hoger et al., 1994; Leib-Mosch et al., 1986).

Activation of these viruses is thought to be associated with osteoma of the skull in mice treated with estradiol or gold thioglucose (Nilsson and Stanton, 1994). Osteosarcomas are characterized by the formation of osteoid by malignant stromal cells and may exhibit a high level of anaplasia and mitotic activity. Lung metastases are common (Long and Leininger, 1999).

Some osteosarcomas (as evidenced by the presence of metastases) can resemble osteoma histologically (Nilsson and Stanton, 1994).

Cartilaginous tumors, chondromas, and chondrosarcomas are extremely rare in mice (Lombard, 1982).

Chondromas are expansile well-circumscribed masses composed of irregular lobules of cartilage, containing well-differentiated but disorganized chondrocytes. Chondromas with areas of osseous metaplasia and/or chondroid formation should be differentiated from osteoma (Long and Leininger, 1999).

Chondrosarcomas are lobulated highly cellular tumors with occasional pleomorphic, binucleate, or multinucleate giant cartilage cells and rare mitotic figures. More anaplastic tumors contain spindle-shaped cells resembling fetal chondroblasts. There is minimal invasion (Ernst et al., 2001b).

Skeletal Muscle

Both neoplastic and nonneoplastic spontaneous lesions of skeletal muscle are uncommon in laboratory mice (Burek et al., 1982; Lombard, 1982; Squire et al., 1978).

Multiple Systems

Amyloidosis

The CD-1 mouse is widely used for chronic toxicology studies. The major cause of death of aged CD-I mice is amyloidosis. Amyloidosis occurs much less frequently in other strains of mice. It is uncommon in B6C3FI mice. The incidence can be increased in association with fighting among group-housed males and with ectoparasitism (Percy and Barthold, 2001).

The disease may be generalized (systemic) or localized. The systemic forms of amyloid may be primary or secondary.

Primary myeloma-associated amyloid comprises fragments of immunoglobulin light chains (AL). Secondary amyloid is derived from serum amyloid A, polymorphic, acute-phase response proteins produced by the liver in response to the release of monokines (including IL-1, and TNF) by macrophages during inflammation.

Secondary amyloidosis can occur spontaneously in laboratory mice, related to chronic inflammation and acariasis.

Generalized amyloid deposits can be seen in many tissues. Amyloid deposition in the small intestine and glandular stomach generally occurs in the lamina propria and submucosa. In the adrenal glands, amyloid deposits are common in the inner cortex surrounding the medulla. Lymph node involvement is primarily in the mesenteric lymph node, and the amyloid deposits occur at the periphery of the node in the subcapsular sinuses. Amyloid deposits in the thyroid and parathyroid involve the interstitium in a diffuse fashion. Amyloid deposits in the kidney primarily involve the glomeruli, but also the interstitium. Splenic perifollicular areas, hepatic periportal areas, pulmonary alveolar septae, male and female reproductive organs, myocardium and aorta can also be involved.

Involvement of the glomeruli of the kidneys is usually the cause of death in animals that die with amyloidosis. Amyloidosis is often associated with atrial thrombosis and left or right congestive heart failure. Mice with amyloid deposits in the renal medullary interstitium can develop papillary necrosis.

Localized forms of amyloid, all with differing composition, are formed in the brain in association with endocrine tumors. Localized amyloidosis of ovarian corpora lutea is frequently seen in CBA and DBA mice in the absence of the systemic disease.

Morphology

Amyloid is a chemically diverse family of proteins characterized by a beta-pleated sheet molecular conformation, which imparts its characteristic staining properties.

With the light microscope and H&E stain, amyloid appears as an amorphous, eosinophilic, hyaline, extracellular substance, which with progressive accumulations encroaching on and producing pressure atrophy of adjacent cells. The most common stain used to confirm amyloid is Congo red, which imparts a pink or red color to amyloid and results in a green birefringence with polarized light. Special stains sometimes used to confirm the presence of amyloid include thioflavin T (secondary fluorescence with ultraviolet light) and metachromasia with crystal or methyl violet.

Genetically Engineered Mice in Toxicology

For many years, the 2-year, two-species (rat and mouse) bioassay has been the accepted standard for carcinogenicity studies intended for submission to regulatory agencies. For some time, however, there has been debate in the profession as to the utility of the traditional mouse bioassay. It has been shown that about half of all known human carcinogens are positive in both rat and mouse and that very few human carcinogens have been identified only in the mouse. A number of potential alternatives to identify carcinogens have been suggested and explored. These include fish models, structure–activity relationships, artificial intelligence programs, receptor-mediated models, the infant mouse model, the rat liver initiation/promotion model, and genetically engineered mice.

In the past few years, there has been an incredible proliferation of genetically engineered mouse models. These include transgenic mice, in which foreign genetic material is inserted into the mouse genome and remains functional, as well as knockout mice, in which one (heterozygous) or both (homozygous) alleles of a gene of interest are "knocked out." Although most genetically engineered mice are used in basic research, several different models show promise in the area of regulatory toxicology.

The advantages of these genetically engineered mouse models when compared to the 2-year bioassay is that they are faster (due to decreased tumor latency), they are cheaper (fewer animals required with shorter protocols), they require lower exposures, they may minimize strain differences, and they are often targeted toward a specific mechanism of tumor formation. The principal hypothesis regarding the use of genetically engineered mice is that the models represent mice with specific genetic alterations critical to tumorigenesis, but which by themselves are insufficient to produce tumors before the end of a short (generally 6-month) study. Thus, the exposure of these mice to trans-species carcinogens would be expected to result in the rapid induction of compound-specific tumors.

Several large validation studies have been completed in the past several years by the National Institute of Environmental Health Sciences and the International Life Sciences Institute (ILSI) consortium for Alternatives to Carcinogenicity Testing, which included over 50 industrial, government, and academic laboratories in the United States, Europe, and Japan. The ILSI results have recently been published in a supplement to the journal *Toxicologic Pathology* (*ILSI/HESI*, 2001). At this time, several models appear to be the best candidates for use in short-term bioassays, either as replacements for the 2-year mouse bioassay or as additional studies to be used in the weight of evidence for a submission (Mahler et al., 1998; Maronpot et al., 2000).

The three models discussed in the following have all been through one or more validation studies and have been used as part of submission packages for pharmaceuticals and/or chemicals both in the United States and in other countries.

p53+/− *Knockout Mouse*

This knockout mouse has a single wild-type p53 allele and one inactivated allele of p53. p53 is a tumor suppressor gene, which is important in repairing DNA. It has been called the gatekeeper of the genome, which, in the presence of DNA damage, causes arrest of the cell cycle in G1 to allow time for DNA repair. When DNA repair is successful, the cell cycle is allowed to progress. If repair does not occur, cells are directed by p53 to undergo apoptosis. With one copy of p53 inactivated, the p53 heterozygous mice are therefore more susceptible to carcinogens, since it takes only a loss or mutation of the remaining copy of wild-type p53 to result in loss of repair function with subsequent tumor development. p53 mice respond to genotoxic carcinogens by the development of tumors. The p53 heterozygous knockout is most often produced on a C57BL/6 background; however, at least five different strains have been used, resulting in different (strain-related) spontaneous tumors.

Spontaneous tumors include a number of sarcomas: sc sarcomas, osteosarcomas, meningeal sarcomas, and urinary bladder sarcomas, as well as alveolar/bronchiolar adenomas in the lung, granulocytic leukemia, and malignant lymphoma (Mahler et al., 1998).

The sc sarcoma complex generally presents as masses of mesenchymal cells in sheets, fascicles, or whorling patterns. These tumors may differentiate to fibrosarcomas, myxosarcomas, rhabdomyosarcomas, hemangiosarcomas, or they may be so poorly differentiated as to require a diagnosis of "sc sarcoma." They occur spontaneously and have been induced by melphalan administered intraperitoneally, sc injection of test compounds, and by the use of sc microchips used for identification purposes.

Malignant lymphoma has been reported in about 2% of control male and female p53 heterozygous mice. Increased rates of malignant lymphoma in p53 heterozygous mice were associated with exposure to melphalan. The lymphoma is usually thymic in origin, and a thymic mass or enlargement is the most common gross manifestation.

The neoplastic cells are large lymphoblasts with sparse cytoplasm, round to vesicular nuclei, and one or more nucleoli. Mitotic figures are common. The tumor often becomes systemic, with neoplastic cells present in the spleen, lymph nodes, lung, heart, liver, and kidney.

An unusual lesion reported in the p53 knockout mouse is atypical hyperplasia of the thymus. There is an absence of a distinction between thymic cortex and medulla. One or both lobes of the thymus may be affected. The lobes are smaller and contain sheets of large atypical lymphocytes, which appear neoplastic mixed with smaller, more normal lymphocytes. There is a proliferation of this mixed-cell population, but it is characteristic that it does not extend beyond the capsule. It has been suggested that affected thymuses first undergo atrophy, followed by a proliferation of remaining cells. This lesion has been induced by several unrelated compounds and is considered to be a preneoplastic precursor lesion of thymic lymphoma.

Tg.AC Mouse

The Tg.AC transgenic mouse uses the FVB/N background and has a mutated Harvey-ras oncogene, with multiple copies on chromosome 11. The expression of this transgene is inducible by carcinogens. The transgene expresses in the skin, which is genetically initiated by the presence of the transgene. The induction of multiple squamous papillomas following application by skin painting represents the reporter phenotype.

There are many spontaneous tumors present in Tg.AC mice at 6 months of age. Significantly, the incidence of skin papillomas is around 4%, so that the presence of papillomas is a reliable indicator

of a positive response. Other spontaneous tumors include papillomas of the forestomach, and odontogenic tumors, which may present as ameloblastomas with anastomosing cords of squamous cells surrounded by loose undifferentiated stroma, or odontomas with abortive tooth structures formed by enamel, dentin, and well-differentiated ameloblasts and odontoblasts. There are also benign and malignant alveolar/bronchiolar tumors in the lung and a carcinoma of the duct of the submandibular salivary glands, which grossly resembles the tooth tumors and often replaces the salivary gland with a cystic structure lined by well-differentiated non-keratinizing stratified squamous epithelium. Systemic neoplasms include erythroleukemia, and malignant lymphoma, which generally arises in the thymus and presents as a well-differentiated lymphocytic lymphoma. With the exception of the odontogenic tumors and the forestomach papillomas, all of these tumors are present at an extremely low incidence (<5%).

Induced tumors are present at the site of application and consist primarily of multiple squamous papillomas. The original intention with the Tg.AC mouse was to count the papillomas, without any additional pathologic evaluation. Generally speaking, the number of papillomas and the time of onset are recorded up to 30 tumors. Occasionally, these tumors present as keratoacanthomas or transform from papillomas to squamous cell carcinomas. A few cases of metastasis have been reported.

In addition to the induction of skin papillomas, it has been shown that carcinogens can induce an increase in the number and size of squamous papillomas of the forestomach, as well as the incidence of malignant lymphoma. An unusual lesion described as myelodysplasia has also shown to be induced by some chemicals. This lesion presents as a wide spectrum of changes in the liver and spleen, and may resemble exuberant EMH, granulocytic leukemia, or lesions that have characteristics of both. As the liver involvement becomes more severe, there is periductular hyaline degeneration, bile duct proliferation, and perivascular fibrosis.

rasH2 Transgenic Mouse

This genetically engineered mouse was developed in Japan. This is the only transgenic mouse used in short-term bioassays that has inserted human genetic material. Based on a CB6F1 background, it has normal coding of the human c-Ha-ras gene, with a mutation in the last intron. There is an overexpression of the gene in both normal and neoplastic tissues. There are few lesions present at 6 months of age. The suggested advantages of this transgenic mouse are that it carries a human oncogene, has more rapid onset of tumors and develops more tumors than in a traditional bioassay, and the tumor types correspond to those present in traditional mouse bioassays.

Spontaneous tumors in the rasH2 mouse include forestomach papillomas and squamous cell carcinomas, alveolar epithelial neoplasms, thymic lymphomas, and splenic hemangiosarcomas (Mitsumori et al., 1998). The most common spontaneous tumors are hemangiosarcomas, which have been reported in the spleen, uterus, and sc tissue. These tumors are of the endothelial type with neoplastic endothelial cells surrounding blood-filled vascular spaces as well as solid cellular masses. Mitoses are common, and there is a delicate to broad-banded collagen stroma in the tumor. Typical transitional cell carcinomas were present in the bladder of rasH2 mice following administration of p-cresidine, a potent bladder carcinogen.

METABOLISM

Shayne Cox Gad

The mouse is the most extensively used experimental animal in biomedical research. In contrast to its popularity as an animal model, information on xenobiotic metabolism in the mouse is much less available compared with information in the rat and dog. This makes proper assessment of the

suitability of this species for predicting xenobiotic metabolism in humans difficult. Although the metabolic patterns of some xenobiotics in the mouse are similar to those in the rat, there are many examples where xenobiotic metabolism is quantitatively and qualitatively different between these species, resulting in a difference in toxicity. The mouse is, in general, a more active oxidizer compared to the rat. Sex differences in xenobiotic metabolism are much less frequently observed in the mouse than in the rat. The development of humanized mouse cell lines has advanced to the point that such mice are a much more attractive model of human drug metabolism and toxicological risk assessment (Cheung and Gonzalez, 2008).

Kato (1966) studied species differences in cytochrome P-450 content in relation to the activity of hepatic microsomal mixed-function oxidase. Among the species studied (male and female mice, male and female rats, male rabbits, and male cats), the mouse has the highest content of cytochrome P-450 with the highest activity of NADPH oxidase, aminopyrine N-demethylase, and aniline hydroxylase (Bell et al., 1993; Kato, 1979; Soulaili-el Amri et al., 1986, 1988). The mouse has the highest liver/body weight ratio (66.2 ± 2.8 g/kg body weight) among the commonly used laboratory animals (e.g., rat, 40.4 g/kg; guinea pig, 40.2 g/kg; rabbit, 34.5 g/kg; dog, 23.2 g/kg), but the microsomal protein concentration in the mouse liver (22.3 mg/g liver) was similar to that of the other species (Gregus et al., 1983). A wide range of drugs induce P450 CYPs, particularly CYP1A (more in humans than rats) CYP2B, CYP3A, and CYP4A. Because of the high concentration in the liver, induction is primarily of hepatic forms (Graham and Lake, 2008). This is a significant factor to the greater sensitivity of mice to liver tumorigenesis. Consequently, the amount of cytochrome P-450 (0.5–1.1 nmol/mg) in the mouse (Table 2.24) was similar to that in the rat and guinea pig when calculated on the basis of milligrams of protein (Chhabra et al., 1974; Litterst et al., 1975; Gregus et al., 1983). However, the value in mouse liver was slightly higher than that in the dog (0.347 nmol/mg). The level of NADPH-cytochrome c reductase activity (109 ± 33 nmol/mg/min) was much lower in the mouse compared with the rat (187 ± 51 nmol/mg/min). The activities in mixed-function oxidase measured by hydroxylase activity for aniline and biphenyl were about two-fold higher in the mouse than in the rat. Aminopyrine N-demethylase activity was similar in these species. In contrast, the activities of benzo(a)pyrene hydroxylase and ethylmorphine *N*-demethylase in the mouse were about half the activities in the rat (Chhabra et al., 1974). Table 2.25 summarizes CYP 450 isoenzymes characterized in the mouse.

Table 2.25 summarizes known P450 CYP isoenzyme activities in the laboratory mouse.

The distribution pattern of mixed-function oxidases for xenobiotics between the smooth and rough microsomal membranes of the liver is known to be species dependent. In the mouse liver microsomes, concentrations of all components of a mixed-function oxidase system were approximately the same between smooth and rough membranes (Gram et al., 1971), whereas in some animals (e.g., monkey, guinea pig), the concentrations were approximately twofold higher in the smooth membrane.

It has been shown that the levels of mixed-function oxidase are generally highest in the mammalian liver compared with the levels in other organs. In the mouse, the content of cytochrome P-450 was approximately fourfold and twofold higher in the liver than in the lung and kidney, respectively. Activity of NADPH-cytochrome c reductase was similar in these organs. Hydroxylase activity for aniline and biphenyl and aminopyrine *N*-demethylase activity were much higher in the liver than in the lung and kidney. However, beta-glucuronidase activity for phenolphthalein in the mouse spleen was 3- to 10-fold higher than that in the liver depending on the strains.

Sex difference in the activity of microsomal mixed-function oxidase is a peculiar phenomenon that is observed clearly in the rat with higher activity in the male than in the female. However, sex differences in mice are not consistently observed (Davis et al., 1969; Kato, 1974; Westfall et al., 1964) and appear to be strain dependent. There was no sex difference in the contents of cytochrome P-450, NADPH-cytochrome c reductase, or NADPH oxidase in the mouse. Sex differences were not evident in aminopyrine *N*-demethylase and aniline hydroxylase activities (Souhaili-El Amri et al., 1987),

Table 2.24   Summary of Hepatic Xenobiotic Drug Metabolizing Enzymes in Mice

Enzyme	Concentration or Activity	Comments and References
Cytochrome P-450 (nmol/mg protein)	1.1,[a] 0.48[b] 1.08 ± 0.05 for M and 1.04 ± 0.07 for F,[c] 0.6 ± 0.02[d]	[a]Chhabra et al. (1974) [b]Souhaili-el Amri et al. (1986) [c]Kato (1979) [d]Flyn et al. (1972) determined at 27°C for 1 h
Cytochrome b (nmol/mg protein)	0.3 ± 0.01,[d] 0.543 ± 0.069[e]	[d]Flyn et al. (1972) determined at 27°C for 1 h [e]Gregus et al. (1983)
NADPH: Cytochrome c reductase (nmol/min/mg protein)	113.0,[a] 127.5,[b] 109,[f] 28.0 ± 1.4[d]	[a]Chhabra et al. (1974), [b]Souhaili-el Amri et al. (1986), [f]Litterst et al. (1975) [d]Flyn et al. (1972) determined at 27°C for 1 h
NADPH: Cytochrome P-450 reductase (nmol/min/mg protein)	1.43 ± 0.18[d]	[d]Flyn et al. (1972) determined at 27°C for 1 h
Hydroxylase (nmol/min/mg) Aniline hydroxylase	16.1 ± 0.1,[a] 1.5,[f] 1.21 ± 0.06 for M and 1.18 ± 0.06 for F,[c] 0.53 + 0.02[d]	[a]Chhabra et al. (1974), [f]Litterst et al. (1975), [c]Kato (1979) [d]Flyn et al. (1972) determined at 27°C for 1 h
Benzo[a]pyrene hydroxylase	114 ± 11 unit/mg/min[a]	[a]Chhabra et al. (1974)
Biphenyl-4-hydroxylase	6.35 ± 0.67[a] 2.8[f]	[a]Chhabra et al. (1974) [f]Litterst et al. (1975)
Hexobarbital hydroxylation	1.51 ± 0.10 for M and 1.53 ± 0.07 for F[c]	[c]Kato (1979)
Styrene 7,8-oxide hydrolase Ethoxycoumarin O-demethyl	1.4[g]	[g]Oesch and Wolf (1989)
N-Demethylase (nmol/min/mg) Aminopyrene N-demethylation	11.0 ± 1.9,[f] 2.33 ± 0.14[d]	[f]Litterst et al. (1975), [d]Flyn et al. (1972) determined at 27°C for 1 h
Ethylmorphine N-demethylation	6.75 ± 0.64[a]	[a]Chhabra et al. (1974)
N-hydroxylase dibenzylamine	0.627 ± 0.0.13[h]	[h]Beckett and Gibson (1975)
UDP-glucuronosyl transferase (nmol/min/mg)		
1-Naphthol	2.6[e]	[e]Gregus et al. (1983)
4-Nitrophenol	6.1[e]	[e]Gregus et al. (1983)
Glutathione S-transferase (nmol/min/mg)		
1-Chloro-2, 4- dinitrobenzene	617g (Cytosol), 147–15[g,i] (Microsomes)	[g]Oesch and Wolf (1989), [i]Morgenstern et al. (1984)
Herachloro-1,3-butadiene	0.109g (Cytosol),	[g]Oesch and Wolf (1989)
Protein estimates		
Microsomal (mg/g)	34.3 ± 9.4[f]	[f]Litterst et al. (1975)
	22.3 ± 1.3[e]	[e]Gregus et al. (1983)
Cytosolic (mg/g)	79.0 ± 3.2[e]	[e]Gregus et al. (1983)

Table 2.25   CYP Activity in the Mouse

7-Ethoxyresorufin *O*-dealkylation	1A1/2, Ryan and Levin (1990)
7-Methoxyresorufin *O*-dealkylation	1A1/2
Caffeine 3-demethylation	1A2, Fuhr et al. (1992); Casley et al. (1997)
Benzphetamine *N*-demethylation	2D10, 2B10, 2C29, Wong et al. (1989); Honkakosk et al. (1992)
7-Benzoxyresorufin *O*-dealkylation	2B10
7-Pentoxyresorufin *O*-dealkylation	2B10, Honkakoski et al. (1992); Honkakoski et al. (1997)
Coumarin 7-hydroxylation	2A5, Jung et al. (1985)
7-Ethoxy-4-trifluoromethylcoumarin deethylation	
Ethoxycoumarin *O*-dealkylation	1A, 2B, Riley et al. (1993)
Tolbutamide methyl-hydroxylation	2C29, Koop (1992)
Chlorzoxazone 6-hydroxylation	2E1, Koop et al. (1985)
4-Nitrophenol hydroxylation	2E1, Koop et al. (1985); Adas et al. (1999)
N-Nitrosodimethylamine *N*-demethylation	2A5
Androstenedione 15α-hydroxylation	
Androstenedione 16α/β-hydroxylation	
Dextromethorphan *O*-demethylation	
Dextromethorphan *N*-demethylation	
Testosterone → Androstenedione	
Testosterone 2α-hydroxylation	2D, Sharma and Shapiro (1995)
Testosterone 2β-hydroxylation	3A11/13, Liu et al. (1995); Yanagimoto et al. (1992)
Testosterone 6β-hydroxylation	3A11/13, Liu et al. (1995); Yanagimoto et al. (1992)
Testosterone 7α-hydroxylation	
Testosterone 15α-hydroxylation	2A4, Iwasaki et al. (1993); Nelson et al. (1993)
Testosterone 15β-hydroxylation	
Testosterone 16α-hydroxylation	2D9, Harada and Negishi (1984); Nelson et al. (1993); Noshiro et al. (1988)
Testosterone 16β-hydroxylation	2B10, 2C29, Honkakoski et al. (1992)
Lauric acid 11-hydroxylation	2E1, Adas et al. (1999)

whereas clear sex differences were demonstrated in the hexobarbital hydroxylase (Castro and Gillette, 1967; MacLeod et al., 1986) and *N*-demethylation activity of ethylmorphine (Catz and Yaffe, 1967; Kato and Onoda, 1966; MacLeod et al., 1987) with significantly higher maximal velocities (V, a) in female than in male mice of some strains. UDP-glucuronyltransferase activity in the male mouse was similar to or significantly greater than that in the female depending on the strain of the mouse (Boutin et al., 1984).

Strain differences in the metabolism of xenobiotics are frequently observed in the mouse as also observed in the rat and rabbit. For example, a marked strain difference in the oxidative metabolism of hexobarbital was observed in mice. The duration of sleeping time after a single dose of the drug, which is determined by the rate of its oxidation, ranged from 20 min in the CS7BL mouse strain to 60 min in the 129J strain. Interestingly, there appears to be an inverse relationship between toxicity and sleeping time in the strains of mice studied. This relationship in time correlates with the strain-dependent rates of hepatic metabolism, perhaps suggesting an intermediate toxic product.

The mouse is known to have a high activity of oxidative enzymes compared with other laboratory animals and humans. The biological half-life of oxidative metabolism of phenobarbital in the mouse is 20–60 min, whereas the half-lives in the rabbit, rat, dog, and human are 60, 140, 260, and 360 min, respectively.

N-Dealkylation is most frequently used by the dog and human and least frequently used by the rat. The mouse uses this pathway for many compounds and appears to have high *N*-dealkylase activity in general. For example, the mouse has been shown to excrete major amounts of the *N*-dealkylated

metabolite of tiaramide (Schwarz et al., 1973), whereas the other species (e.g., rat, dog, and monkey) favor *N*-oxide formation and/or side chain oxidation. Oxaminase has been shown to be metabolized by *N*-dealkylation in the mouse but not in the rat. When *N*-demethylation activity for aminopyrine was examined in the mouse, rat, rabbit, and cat, the enzyme activity in the mouse was highest and at least threefold higher than that in the male rat (Kato, 1966). In addition, there was no sex difference in the *N*-demethylase activity in the mouse, whereas the enzyme activity in the male rat was approximately fourfold higher than that in the female rat. Castro and Gillette (1967) studied species difference in *N*-demethylation of ethylmorphine using hepatic microsomes of the mouse, rat, guinea pig, and monkey. Ethylmorphine *N*-demethylase activity in the oak and female mouse was higher than that in the other species except for the male rat. *N*-Demethylase activity in the male mouse (V_{max} of 139 mmol/mg/10 min) was approximately half the activity in females. Later, Van Den Berg et al. (1977) found that ethylmorphine *N*-demethylase activity differed between the sexes in one strain of mouse (PB-SE) but not in another strain (CPB-V). In the rat, the ethylmorphine *N*-demethylase activity in males (V_{max} of 203 mmol/mg/10 min) was approximately fivefold higher than in females.

Diazepam is metabolized oxidatively by two metabolic pathways: one is ring hydroxylation, which is the main pathway in the rat, and the other is oxidative *N*-demethylation, which is the main pathway in the mouse and man. Although concentrations of diazepam in blood and brain are similar in the mouse and rat, antimetrazol activity is longer lasting in the mouse than in the rat owing to the accumulation of active *N*-demethylated metabolite in the mouse brain (Marcucci et al., 1968). In humans, the *N*-demethylated metabolite was the major metabolite detected in plasma.

Amphetamine is metabolized oxidatively by two metabolic pathways: one is ring hydroxylation and the other oxidative deamination (Dring et al., 1966). *N*-deamination of amphetamine occurs in the mouse as in humans (Caldwell, 1976). In the rat, amphetamine was shown to be extensively metabolized by aromatic hydroxylation and poorly metabolized by oxidative deamination. Based on the available metabolism data, the mouse appears to be a relatively good animal model, in general, for *N*-dealkylation and *N*-deamination. However, the extent of *N*-dealkylation and *N*-deamination is highly compound dependent, and caution must be exercised for this generalization.

There are several microsomal aromatic hydrocarbon hydroxylases (AHHs) whose occurrence depends not only upon species but also on the nature of the aromatic compound. Generally, the activities of these enzymes are much greater in the mouse than in humans as observed with amphetamine and benzo(a)pyrene (Souhaili-el Amri et al., 1986). Aromatic hydroxylation of amphetamine occurred to the extent of 10%–19% in the mouse and about 3% in humans. In the rat, it was about 60% (Parke, 1968). In contrast to amphetamine hydroxylase activity, the activity of benzo(a)pyrene hydroxylase in the mouse was approximately threefold higher than that of the male inbred SD rat (Oesch et al., 1973). The AHH activity in human liver was approximately 30%–60% of the hepatic activity of the male rat (Pelkonen et al., 1975). Although the bezo(a)pyrene hydroxylase activity was similar in male and female mice, a sex difference has been shown in the rat, with the female displaying about 20%–40% of the hepatic activity of the male.

Biphenyl was metabolized in the mouse to 2-hydroxybiphenyl as well as 4-hydroxybiphenyl, which is the metabolite of all species examined (Creaven et al., 1965). However, in rats, the 2-hydroxy metabolite was formed only in young animals but not in the adults, although rats are known to be a good animal model for aromatic hydroxylation. Therefore, the distribution and the activity of the microsomal hydroxylases among species cannot be predicted for all compounds. If a difference is found among species with a given compound, then it is likely, but not always certain, that a similar species difference may consistently occur with its derivatives (Williams, 1974).

Many unreactive compounds may be metabolized to chemically highly reactive intermediates and act as mutagenic/carcinogenic agents. Unreactive aromatic hydrocarbons and olefinic compounds are converted to highly active arene oxides and alkene oxides that are formed from the epoxidation of double bonds in aromatic rings and olefinic double bonds, respectively. The mouse is reported to have high epoxygenase activity (Gregus et al., 1983). In a comparative *in vitro* metabolism

study of naphthalene using microsomal preparations from lung, liver, and kidney of mice, rats, and hamsters, it has been shown that the metabolism of naphthalene to an epoxide was most extensive with microsomal preparations from the mouse lung (Buckpitt et al., 1987). Furthermore, metabolism of naphthalene was stereoselective to form (IR,2S)-naphthalene 1,2-oxide with the mouse lung, which is the target tissue for acute toxicity in this species. In contrast, with microsomal preparations from the mouse liver and kidney, and with all microsomal preparations from the rat and hamster, metabolism was not stereoselective.

The epoxides are metabolized to much less reactive vicinal diols by epoxide hydrolases and to glutathione conjugates by glutathione S-transferase. Therefore, the level and the activity of epoxide hydrolases among species may be of great importance for the mutagenic/carcinogenic risks. With styrene oxide as the substrate, the epoxide hydrolase activity in the mouse (1.4 nmol/mg protein/min) was less than one-fortieth of that in humans (59.3 nmol/mg protein/min) (Oesch and Wolf, 1989). However, in the diabetic mouse (male and female db/db strain), the styrene oxide hydrolase activity was increased approximately threefold (Watkins and Klueber, 1988). In addition, genetic polymorphism of microsomal epoxide hydrolase activity in the mouse has been reported (Lyman et al., 1980).

A species, such as mice, having high epoxygenase activity (Gregus et al., 1983) but low epoxide hydrolase activity (Oesch and Wolf, 1989) may be much more susceptible than the human to toxicity related to epoxide formation. However, epoxides are also detoxified via glutathione conjugation. The mouse liver has a high level of glutathione transferase activity (149 ± 13 nmol/mg/min) compared to that of rats (87 ± 10 nmol/mg/min) and humans (25 ± 4 nmol/mg/min) (Pacifici et al., 1981). For example, following acrylonitrile administration, concentrations of an oxide metabolite (2-cyanoethylene oxide) in rat blood were 6–11 times higher than those in the mouse, although mouse liver oxidized acrylonitrile to the oxide at a much greater rate (approximately three times) than rat liver (Kedderis, 1989). Acrylonitrile was metabolized to thiodiglycolic acid, a metabolite of the glutathione conjugate, seven times faster in the mouse than in the rat. Therefore, to what extent these interspecies differences in the epoxide toxicity will occur for other substrates remains to be further evaluated.

It has been reported that N-hydroxylase activity is predominately mediated via the polycyclic hydrocarbon-inducible PI-450 (Felton et al., 1976) and is of great importance for toxicity evaluation. The mouse is the most susceptible to acetaminophen-induced hepatic injury, and the rat had the lowest susceptibility. This difference in toxicity was found to be related to the rates of *N*-hydroxylation of the drug by the hepatic microsomes (Davis et al., 1974). An increase in *N*-hydroxylation enhances the need for reduced glutathione, and glutathione depletion in the liver precedes marked increases in covalently bound acetaminophen. Formation of metabolites covalently bound to microsomal protein and depletion of hepatic glutathione were highest in the mouse, but only minor extents of covalent binding and depletion of glutathione were observed in the rat. As a result, in the mouse, acetaminophen induced hepatotoxicity. Increases in covalently bound metabolites of the drug were found to be highly correlated with the Ali' allele (Thorgeirsson et al., 1977).

Lotlikar et al. (1967) studied species differences in the relative N- and ring hydroxylation of 2-AAF by liver microsomes of the mouse and other species. The *N*-hydroxylase activity in the mouse was lower than that in hamster and rabbit but higher than that in the rat and guinea pig. The *N*-hydroxy metabolite was practically undetectable in the guinea pig, which explains resistance of the guinea pig to hepatoma induction by 2-AAF. Later, Razzouk and Roberfroid (1982) reported that V_{max} values for *N*-hydroxylase of aminofluorene and AAF in the mouse (510 and 225 pmol/mg/min, respectively) were higher than those in the hamster (260 and 140 pmol/mg/min, respectively) and at least 10-fold higher than those in the rat (41 and 14 pmol/min/min, respectively). *N*-Hydroxylase activities were also studied in several species with dibenzylamine (Beckett and Gibson, 1975) and 4-aminobiphenyl (McMahon et al., 1980). In contrast to AAF *N*-hydroxylase activity, *N*-hydroxylase activity of dibenzylamine (18.8 nmol/mg/30 min) and 4-aminobiphenyl (13.2 gmol/80 min) in the

mouse was approximately twofold lower compared with guinea pig liver. The activity of dibenzyl-amine N-hydroxylase in the mouse was slightly higher than that in the rat (12.5 nmol/mg/30 min), whereas 4-aminobiphenyl N-hydroxylase activity in the mouse was fivefold higher than that in the rat (2.4 gmol/80 min). Thus, based on the limited information available, N-hydroxylase activity in the mouse is moderate and similar to or higher than that of the rat.

The mouse is not reported to display any defects in conjugation reactions such as those observed in the cat (glucuronidation), pig (sulfation), and dog (acetylation). In acetylation of certain aromatic amines, both fast and slow acetylators have been identified with some mouse strains such as C57BL/6J and A/J as with the human population (Elves et al., 1985). Lower and Bryan (1973) studied acetylation of the carcinogens 2-aminofluorene, 4-aminobiphenyl, and 2-aminonaphthalene by the mouse liver cytosol enzymes. The most readily acetylated carcinogen was 2-aminofluorene (332 ± 25 nmol/50 mg liver/5 min), followed by 4-aminobiphenyl (215 ± 15 nmol/50 mg liver/5 min) and 2-aminonaphthalene (209 ± 9 nmol/50 mg liver/5 min). These enzyme activities in the mouse liver were about half the activities in the hamster liver but 6–10 times higher than those in the rat liver. Recently, Calabrese (1988) reported that the mouse was about 4.6 times more efficient acetylator than the human fast acetylator for 2-aminofluorene. The rat displayed about half the activity of the human fast acetylator and sevenfold greater activity to acetylate 2-aminofluorene than the human slow acetylator, which is consistent with previous findings (Lower and Bryan, 1973).

Lower and Bryan (1976) also studied deacetylase activities of the acetylated derivatives of each of the earlier same three compounds in the mouse, hamster, rat, guinea pig, and dog. Deacetylase activity of 4-acetylaminobiphenyl was highest in the mouse and more than 20 times that observed in the rat. However, deacetylase activities for 2-AAF (54.7 ± 6.3 nmol/mg/h) and 2-acetylaminonaph-thalene (21.1 ± 6.3 nmol/mg/h) in the mouse were approximately three- and sixfold lower compared with those of hamster liver. The guinea pig and rat were generally the least efficient in the deacety-lation of these compounds. The relative capacity of the animal model to acetylate and deacetylate is an important variable with respect to arylamine-induced cancer. In the mouse, as in the rat, the ratio of N-acetyltransferase to deacetylase activity is very high relative to the dog. This may provide rodents with some protection against bladder cancer, but not hepatocarcinogenesis.

Surprisingly, there is little information available for glucuronidation activity of drugs in the mouse. Based on limited information, UDP-glucuronosyltransferase activities in rodents and pri-mates are generally high compared with that in the cats (Caldwell, 1978). Gregus et al. (1983) stud-ied hepatic UDP-glucuronosyltransferase activity toward I acceptors in the mouse, rat, guinea pig, rabbit, cat, quail, and trout. The transferase activity in the mouse, when compared to the activity in other species, was high toward phenolphthalein, diethylstilbestrol, morphine, valproic acid, and bilirubin, whereas the activity was medium or low toward 1-naphthol, p-nitrophenol, testosterone, estrone, chloramphenicol, and digitoxigenin. Therefore, the UDP-glucuronyltransferase activity appears to be highly substrate specific, and no general pattern in its activity could be established among the species studied. Considerable evidence has accumulated, which suggests that multiple forms of UDP-glucuronyltransferase exist, and this may explain why glucuronidation activity is high for some substrates but not for others.

A strain difference in UDP-glucuronosyltransferase activity in the mouse has been reported (Tsyrlov and Lyakhovich, 1978). After pretreatment with 3-methylcholanthrene, the microsomal UDP-glucuronosyltransferase activity increased threefold in C57BL mice but not in AKR mice, although the transferase activity in the control animals was similar between these two strains.

Hydrolysis of glucuronide conjugates is carried out by the lysosomal enzyme beta-glucuronidase, which is present in most tissues, particularly liver, kidney, spleen, intestine tract, endocrine, and reproductive organs. The level of beta-glucuronidase in multiple tissues has been studied in a variety of animal species as well as in humans. Genetic polymorphism of beta-glucuronidase activity was observed in the various tissues of the mouse in which the activity in the high-activity strain is 4–10 times greater than that of the low-activity strain. The enzyme levels in the liver, kidney, and spleen

of the high-activity strain of the mouse were similar to the activity in the respective human tissues, whereas the activities in the rat were approximately 10-fold higher than those in humans. The beta-glucuronidase of the cecal contents and feces was also similar in mice and humans (Rowland et al., 1986). However, beta-glucuronidase activity in the mouse intestine was much higher than that in humans and rats. The activities of beta-glucuronidase in the spleen were similar to or higher than those in the liver regardless of species and strain of mouse.

As discussed earlier, GSH transferase activity in the mouse liver was reported to be higher than that in the rat with 1-chloro-2,4-dinitrobezene as a substrate (Igarashi et al., 1983, 1986) and also higher than human liver with styrene oxide as a substrate (Pacifici et al., 1981). Recently, Oesch and Wolf (1989) determined glutathione transferase activities in liver microsomes and cytosol from various species, including mice and humans with hexachloro-1,3-butadiene and 1-chloro-2,4-dinitrobenzene. Contrary to the earlier findings, the glutathione transferase activities in the mouse were lower than those in the rat with the same substrate and also lower than those in humans. This difference may be, in part, due to strain difference of the mouse and substrate difference in humans among the studies. The transferase activity in the microsomes (0.16 nmol/min/mg) and cytosol (0.109 nmol/min/mg) was similar in the mouse, whereas in humans, the enzyme activity was much greater in microsomes (1.17 nmol/min/mg) than in cytosol (0.031 nmol/min/mg) with hexachloro-1,3-butadiene as a substrate (Oesch and Wolf, 1989). However, with 1-chloro-2,4-dinitrobenzene, GSH transferase activity in the cytosol fraction in mice (617 nmol/min/mg) and humans (1666 nmol/min/mg) was about 11- and 30-fold higher than the activities in the microsomal fraction, respectively. Therefore, the GSH transferase activity appears to be highly compound specific, and it is difficult to make any broad generalization.

Thiol methylation is an important pathway in the metabolism of many sulfhydryl residues. At least two enzymes, thiol methyltransferase (TMT) and thiopurine methyltransferase (TPMT) can catalyze the thiol methylation. TMT is membrane associated and catalyzes the S-methylation of aliphatic sulfhydryl compounds such as 2-mercaptoethanol, captopril, and D-penicillamine. It is inhibited by SKF 525A but not by the benzoic acid derivatives that are potent inhibitors of TPMT. Ottemess et al. (1986) measured TMT activity in hepatic microsomes from 10 different strains of mice using 2-mercaptoethanol as the methyl acceptor substrate and compared the activity with that in human red blood cell membranes and kidney microsomes. Since the properties of TMT in the mouse liver were similar to those of the enzyme in the human tissues, the authors concluded that the inbred mouse is a useful experimental animal model to study the action and function of TMT.

Very little work has been reported on the conjugation reactions of xenobiotics with stiffate, taurine, and amino acids in the mouse. Based on the limited information, the mouse appears to have moderate sulfate conjugation capability as illustrated with acetaminophen and 4-hydroxy-3-methoxyphenyl ethanol. The concentration of adenosine 3′-phosphate-5′-phosphosulfate (PAPS), which is required for sulfation reactions as the sulfate donor, in the male mouse liver (29.4 ± 1.6 mmol/g of tissue) was lower than that of rats (67.9–76.8 mmol/g), similar to hamsters and rabbits, but much higher than that in the dog (16.1–17.3 mmol/g). The concentrations of PAPS in the mouse kidney, lung, and intestine were approximately half that of liver.

Emudianughe et al. (1978, 1987) studied amino acid conjugation of the isomeric naphthylacetic acids in seven subprimate animal species, including the mouse. With 2-naphthylacetic acid, the taurine conjugate was the major urinary conjugated metabolite (57%), whereas the glucuronide conjugate was 40% of the urinary conjugated metabolites. However, with 1-naphthylacetic acid, the majority of the urinary conjugate metabolites in the mouse was the glucuronide (68%), and the glycine conjugate was minor (16%). In general, the dog appears to be the most extensive taurine conjugator, man and rhesus monkey are weak species for this conjugation pathway, and the mouse is intermediate. An exception to this is the conjugation metabolism of an arylacetic acid, oxepinac. Oxepinac was metabolized to the taurine conjugate in the mouse as well as in the rat (Hakusui et al., 1978). However, in the dog and man, it was conjugated with glucuronic acid, although the dog is

known to be an extensive taurine conjugator with some other compounds. Glutamine conjugates of naphthylacetic acids were not detected in the mouse urine, although other rodents such as the rat and hamster formed substantial amount of this conjugate.

The drug-metabolizing enzymes in the mouse are generally induced or inhibited with compounds known to be enzyme inducers or inhibitors, respectively, in the other species. An exception to this is that DDT, an enzyme inducer for the rat, pig, and sheep, did not produce any enhancement of the oxidative metabolism of drugs in the mouse (Hart and Fouts, 1965; Gabliks and Maltby-Askari, 1970). Furthermore, with some inducers, marked strain differences were observed in the induction of xenobiotic metabolism in the mouse (Glass and van Lier, 1988). A typical example includes AHH induction by polycyclic hydrocarbons. The AHH assay is a reliable, simple, and very sensitive assessment of aromatic hydrocarbon responsiveness following treatment of animals with polycyclic hydrocarbon inducers. Using AHH induction as an indicator of phenotype at the Ah locus, several laboratories have found that about half or slightly more than half of all inbred mouse strains examined were responsive, and the remaining mouse strains were nonresponsive (Nebert et al., 1979).

SKF-525A inhibited aminopyrine N-demethylase and hexobarbital hydroxylase in mouse hepatic preparations as in rat hepatic preparations. However, the inhibition was quasicompetitive in the mouse, whereas in the rat, it was competitive, thus indicating differences in the nature of the monooxygenases between these two species.

Not many in-depth works have been published about stereoselectivity in metabolism and toxicity of xenobiotics in the mouse. One interesting example, however, is stereoselective toxicity of thalidomide. Both S-(-)thalidomide and the R-(+)-enantiomer are transformed into I-N-phthaloylglutamine and I-N-phthaloylglutaminic acid in various species. Although conflicting reports have been published on potential racemization of thalidomide enantiomers and the sensitivity of different mice and rat strains as to thalidomide toxicity, in SWS mice only the glutamic acid metabolite derived from the S-(-)-enantiomer and not the one derived from the R-(+)-isomer was shown to be embryotoxic and teratogenic (Ockenfel et al., 1976).

Species differences in the toxicity of a compound may be due to factors other than metabolism differences. Some of these factors include protein binding, biliary excretion, transporter activity, and differences in absorption and elimination. When protein binding of some drugs (clofibric acid, etodolac, tolrestat, perrinone, benoxaprofen) was examined in the rat, mouse, dog, rabbit, rhesus monkey, and human, the binding was, in general, highest in human serum, weakest in the mouse, and somewhat variable with other species.

Differences in biliary and transporter efflux excretion may have pronounced toxicological implications, especially when followed by reabsorption, i.e., enterohepatic circulation takes place. For example, the intestinal toxicity of indomethacin in five species of laboratory animals (rat, dog, monkey, guinea pig, and rabbit) is inversely proportional to the exposure of the intestinal mucosa to the drug as a consequence of enterohepatic circulation. In general, the mouse, rat, and dog are very efficient in biliary excretion, and guinea pigs, monkeys, and human are relatively inefficient. For example, the biliary excretion rate of methyl mercury was substantially higher in the mouse, rat, and hamster (approximately 0.8 nmol/min/kg) compared with that in the guinea pig and rabbit (0.15 and 0.03 nmol/min/kg, respectively) (Stein et al., 1988). The biliary excretion rate of GSH-related thiols and disulfides was also highest in the mouse. Efflux of xenobiotics by transporters in the mouse kidney is extensive and is hormonally regulated (Alnouti and Klaassen, 2008). At least 37 different portions have been identified in the mouse liver already, and several of these are also found in the lung, testes, ovary, and placenta of the mouse.

As reported for other species, the drug-metabolizing enzymes in newborn mice are not fully developed. When phenobarbital was injected in mice of various ages, the newborn animals failed to metabolize any of the drugs over a 3-hour period. One-week-old mice metabolized about 18% and 3-week-old mice about 22%. A hexobarbital sleeping time study also revealed striking differences with age (Jondorf et al., 1958). The sleeping time of 1-week-old mice was about 107 min, whereas the sleeping time of adult was less than 5 min.

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The Rat

Mark D. Johnson, Shayne Cox Gad, and Christopher J. Kemper

CONTENTS

TOXICOLOGY

Mark D. Johnson

History

    The Norway rat (*Rattus norvegicus*) is believed to have originated in Asia and spread throughout the world with modern civilization as an economic pest. The rat, considered to be the first animal to be domesticated for strictly scientific purposes (Richter, 1959), was first used experimentally in France in the study of adrenal gland function (Philipeaux, 1856, as cited in Lindsey, 1979). Early research with rat in the areas of nutrition, endocrinology, physiology, and behavior led to discoveries such as the nutritional quality of various amino acids in mammals, the existence of vitamins, the characterization of the hormones of the anterior pituitary, and the existence of circadian rhythms.

The rat has become a species of choice for almost every area of biological and medical research because of its size, relatively docile nature, life span, and gestation period.

For the purpose of reducing variability and emphasizing various desirable characteristics, several early researchers established breeding programs. Henry Donaldson and his colleagues at the Wistar Institute (Philadelphia, Pennsylvania) were prominent in this effort. Many of the rat strains commonly used in toxicology today, including the Wistar, Sprague Dawley, and Long-Evans, can be traced to the Wistar lineage. The Fischer 344, another commonly used strain, was developed for use in cancer research at the Crocker Research Institute of Columbia University, New York City.

The use of the rat in toxicology studies has paralleled its use in other fields. The rat continues to be the rodent species of choice for most toxicology studies.

Choice of the Rat in Toxicology Research

Ideally, products intended for use in humans, alongside products that humans could be exposed to, should be tested in humans. The data from humans would apply without reservation to complex human physiology and cellular/biochemical mechanisms and human risk assessment. Unfortunately, humans cannot be used for this purpose. Therefore, the choice of an appropriate species for toxicology studies should be based upon a comparison of the pharmacokinetics (PK) and metabolism of the test compound in different laboratory species and man. In the absence of these data, this choice is often based upon practicality and economics. The rat has metabolic similarities to humans making it a species of choice, alongside the small size, relatively docile nature, short life span, and short gestation period. The extensive use of the rat in research has led to the development of a large historical database of their nutrition, diseases, and general biology.

Species Differences

While the rat is a species of choice in toxicology research because of the many physiological similarities and anatomical characteristics, differences exist that must be considered when designing and conducting studies with this animal. Rats are obligate nose breathers, as such an inhaled test material is subject to nasal filtration and absorption. The placenta is considerably more porous in the rat. This difference may increase the chance of fetal exposure to an administered test material or increase the overall level of fetal exposure to an administered test material. The overall distribution of intestinal microflora is different in the rat, which may lead to differences in the metabolism of an orally administered test material. These and other differences in the rat may lead to positive signs of toxicity to a test material that may not be present in a different species.

Strain Differences

Breeding rats for specific characteristics has produced some physiological differences between strains of rats. Some of these differences are known to affect how the various strains react to toxicants. Among others, strain-specific differences have been found in sensitivity to thiourea (Dieke and Richter, 1945), sensitivity to acetaminophen nephrotoxicity (Newton et al., 1985), the incidence of spontaneous glomerular sclerosis (Bolton et al., 1976), sensitivity to the carcinogenic actions of 7,12-dimethylbenz(a)anthracene (Boyland and Sydnor, 1962), the effects of trimethyltin on operant behavior and hippocampal GFAP (MacPhail et al., 2003), differences in renal carcinogenesis (Hino et al., 2003), differences in cytochrome P4501A1 gene expression caused by 2,3,7,8-tetrachlorodibenzo-p-dioxin in the liver (Jana et al., 1998), susceptibility to 4-nitroquinoline 1-oxide–induced carcinoma (Kitano et al., 1992), and differences in the levels of drug-metabolizing enzymes (Page and Vesell, 1969). In recent years, research and breeding programs have been focused on producing inbred and outbred strains focused on specific disease models and susceptibility to the development of certain carcinoma. When choosing a strain for use, it is important to consider these differences.

Table 3.1 Incidence of Common Spontaneous Tumors in Fischer 344 and CD (SD)IGS Rats

		% Tumors in Untreated Rats							
		CD(SD)IGS		CD (SD)		Fisher		Wistar	
Organ	Tumor Type	Males	Females	Males	Females	Males	Females	Males	Females
Adrenal gland	Pheochromocytoma	10.0	2.3	11.3	2.3	11.9	3.2	3.2	1.3
Mammary gland	Fibroadenoma	1.4	44.5	1.3	16.7	0.8	7.1	1.2	30.2
Pancreas	Islet cell adenoma	3.6	1.4	4.0	0.3	1.5	0.2	5.3	1.9
Pituitary gland	Adenoma pars distalis	33.6	56.8	35.7	50.3	12.4	28.2	41.1	65.8
Testis	Interstitial cell tumor	1.8		7.0		74.6		4.3	
Thyroid gland	C-cell adenoma	10.5	5.0	5.0	5.7	12.5	8.2	10.1	10.7

Sources: Adapted from Charles River, *Spontaneous Neoplastic Lesions in the CRL:CD BR Rat*, Charles River Laboratories, Wilmington, MA, (2001); Mitsumori et al., Variability in the incidence of spontaneous tumors in CD (SD) IGS, CD (SD), F244 and Wistar Hannover rats in Biological Reference Data on CD(SD) IGS Rats, Yokohama, CD(SD) IGS Study Group, 2001.

Of importance for carcinogenicity studies, strain differences have been found in the incidence of spontaneous tumors. Table 3.1 gives the incidence of spontaneous tumors found in commonly used strains in carcinogenicity studies. The historical incidence is important to the analysis of a study in that a high spontaneous rate may mask a small test material–related increase in tumor incidence.

Normal Physiological Values

General values for selected physiological parameters are given in Tables 3.2 and 3.3. Normal values will vary based upon the strain of animal, supplier, feed, and housing conditions. These tables should be used as a point of reference only.

Husbandry

The environment to which a rat is exposed can have profound effects on the result of a research study. Subtle or short-term changes in the environment can alter the response to a test material. Good animal husbandry is essential to maintain the health of the animals on study, increase the reproducibility of the results, and eliminate variable that may confound the results of a study. A good animal husbandry program requires proper oversite of the facility to ensure the health of the animal and takes into account the nutritional and environment needs of the animal.

Facilities

Temperature and Relative Humidity

Current specifications for temperature and humidity are 18°C–26°C and 30%–70% relative humidity (Clough, 1991; ILAR, 1997). These ranges are designed to allow homeotherms to maintain a minimum metabolic rate or to be within their thermoneutral zones (Bligh and Johnson, 1973). Rats have the ability to adapt to changes in temperature and humidity through physiologic, behavioral, or metabolic mechanisms, but this requires time, causes stress to the animal, and may alter the outcome of an experiment. The degree to which an individual is able to adapt is dependent upon the conditions under which the animal is housed, i.e., group housing, type of cage, and bedding.

Table 3.2   Selected Normative Data

Husbandry	
Room temperature (°C)	18–26
Relative humidity (%)	30–70
Ventilation (air change/h)	10
Light/dark cycle (hours)	12–14/12–10
Minimum cage floor size	
Housed individually (cm^2)	350
Breeding with pup (cm^2)	800
Group housed (cm^2 adult)	250
General	
Life span (years)	2.5–3.0
Surface area (cm^2)	0.03–0.06
Chromosome number (diploid)	42
Water consumption (mL/100g/day)	10–12
Food consumption (g/day)	20–40
Average body temperature (°C)	37.5
Reproduction	
Puberty (males and females)	50 ± 10 days
Breeding season	All year
Type of estrous cycle	Polyestrous
Length of estrous cycle	4–5 days
Duration of estrous	10–20 h
Mechanism of ovulation	Spontaneous
Time of ovulation	7–10 h after onset of estrous
Time of implantation	Late day 4 or 5[a]
Length of gestation	21–23 days
Litter size	8–16 pups
Birth weight	5–6 g
Eyes open	10–12 days
Weaning age/weight	21 days/40–50 g
Cardiovascular	
Arterial blood pressure	
Systolic (mmHg)	116–145
Diastolic (mmHg)	76–97
Heart rate (beats/min)	296–388
Cardiac output (mL/min)	10–80
Blood volume (mL/kg)	64
Pulmonary	
Respiration (breaths/min)	100–140
Tidal volume (mL)	1.1–2.5
Compliance (mL/cm H_2O)	0.3–0.9
Resistance (cm H_2O/mL s)	0.1–0.55
Pattern	Obligate nasal
Renal	
Urine volume	15–30 mL/24 h
Na$^+$ excretion	200 mmol/L/24 h
K$^+$ excretion	150 mmol/L/24 h

(*Continued*)

Table 3.2 (*Continued*)   Selected Normative Data

Urine osmolarity	2000 mOsm/kg H_2O
Urine pH	7.3–8.5
Urine specific gravity	1.01–1.07
Urine creatinine	6 μmol/L/24 h
Glomerular filtration rate	1.0 mL/min/100 g body weight

Sources: Baker et al., Housing to control research variables, in *The Laboratory Rat*, Vol. 1, Baker et al. (eds.), Academic Press, New York, pp. 169–192, 1979; Bivin et al., Morphophysiology, in *The Laboratory Rat*, Vol. 1, Baker et al. (eds.), Academic Press, New York, pp. 73–103, 1979; Peplow et al., Parameters of reproduction, in *Handbook of Laboratory Animal Science*, Vol. 1, Melby, E. E. Jr., and Altmon, N. H. (eds.), CRC Press, Boca Raton, FL, pp. 164–183, 1974; Waynforth (1980); Moreau et al. (1998); Sharp, P. E. and La Regina, M. C., *The Laboratory Rat*, CRC Press, Boca Raton, FL, 1998; Van Zutphen et al., *Principles of Laboratory Animal Science*, Elsevier, Amsterdam, the Netherlands, 1993.

[a] The estrous cycle length may vary from 4 to 5 days between strains. Time of implantation may vary based upon the length of the estrous cycle and is dependent upon Day 0 or the first day sperm is found in the vagina.

Table 3.3   Growth Rates in Selected Rat Strains

	Age (days)							
	Crl:CD (SD)IGSBR		Crl:(WI)BR		Crl:(LE)BR		CDF(F-344)/CrlBR	
Weight (g)	M	F	M	F	M	F	M	F
Up to 50	Up to 23	Up to 23	Up to 23	Up to 25	Up to 21	Up to 21	Up to 23	Up to 23
51–75	24–28	24–29	24–28	26–30	22–25	22–26	24–29	24–29
76–100	29–34	30–35	29–32	31–34	26–29	27–31	30–34	30–35
101–125	35–37	36–39	33–35	35–40	30–34	32–36	35–39	36–42
126–150	38–42	40–44	36–40	41–47	35–37	37–43	40–45	43–55
151–175	43–45	45–50	41–44	48–56	38–42	44–50	46–50	56–72
176–200	46–49	51–56	45–48	57–64	43–46	51–55	51–57	73–105
201–225	50–52	57–70	49–52	65–81	47–49	56–69	58–63	105+
226–250	53–56	71–84	53–56	82–105	50–55	70–86	64+	
251–275	57–59	84–105	57–61	106+	56–58	87–102		
276–300	60–65	106+	62–67		59–64	103+		
301–325	66–71		68–73		65–70			
326–350	72–77		74–79		71–80			
351–375	78–87		80–87		81–90			
376+	88+		88+		91+			

Source: Adapted from Charles River, *Background Data on Rat Strains*, Charles River Laboratories, Wilmington, MA, 2004.

The stress caused to the animal during a time of adaptation to changes in the environment has been shown to affect basic physiologic function and behaviors. Animals housed at temperatures below the recommended range have been shown to have increased food intake, increased weight gain, increased energy expenditure, but a decrease in efficiency (Rothwell and Stock, 1986). Conversely, animals housed at temperatures above the recommended range have been shown to exhibit decreased food consumption and body weights (Hamilton, 1967). In addition, male rats develop testicular atrophy consistent with degeneration of the seminiferous epithelium and failure of spermatocyte maturation when housed at temperatures exceeding 26.7°C for more than 48 hours (Pucak et al., 1977). Extremes in temperature have been shown to produce significant differences in body weights, food intake, and hematologic and serum biochemical parameters as well as reproductive parameters across generations of animals housed under these conditions (Romanovsky et al., 2002; Yamauchi et al., 1981).

The influence of temperature on the toxic response has been well reviewed (Clough, 1982; Fuhrman and Fuhrman, 1961; Weihe, 1973). Fuhrman and Fuhrman (1961) postulate three patterns

by which toxicity may vary with temperature: toxicity may increase at temperature extremes; toxicity may increase linearly with temperature; or toxicity may remain constant with increasing temperature to a threshold, and then toxicity begins to increase.

The relative humidity within the colony room environment or with the microenvironment within microisolator cages should be maintained between 50% and 70% for optimal health. While the guide to animal welfare allows for a great range, research has shown that animals housed in relative humidity below 40% may develop lesions such as ringtail and food consumption may be elevated (Clough, 1982; Fox et al., 1984).

Lighting

Appropriate lighting and light cycle play a key role in maintaining the physiology and behavior of the rat. Lighting within the animal colony should be of a sufficient level to provide for animals well-being and allow for animal care activities and safe working conditions for the animal care staff. Light in the animal rooms should provide for adequate vision and for neuroendocrine regulation of diurnal and circadian cycles (Brainard (1989) as cited in the Guide for the Care and Use of Laboratory Animals (1997)). The current guideline for the laboratory rat is that levels be 325 lux approximately 1 m above the floor (ILAR, 1997). This level of light is considered to be adequate for the performance of animal care activities with a limited risk for the development of retinopathies (Bellhorn, 1980).

Variations in light intensity should be taken into consideration when arranging animals on cage racks for toxicology studies. There can be as much as an 80-fold difference in light intensity between a polycarbonate box positioned at the top of a rack and one positioned at the bottom (Weihe et al., 1969). This difference can be even greater when using wire mesh caging and should be taken into consideration when planning long-term subchronic, chronic, and carcinogenicity studies. It is good practice to arrange animals on the caging rack such that dose groups are placed sequentially in vertical order and cage position is rotated periodically (monthly). This will allow for randomization and control of possible retinopathies that may occur over the life of the study.

Most research facilities operate within a 12-hour light/12-hour dark cycle, but a 14-hour light/10-hour dark cycle is also acceptable. Maintenance of an appropriate light dark cycle for the rat is important for the health and well-being of the animals. The rat is a nocturnal animal and is more active at night. Often, feeding studies are conducted on a reverse light dark cycle that requires acclimation of the animal prior to the start of a study. The reversal of the light dark cycle allows for the performance of study function during normal working hours.

It is important that monitoring procedures are in place to assure that the light cycle is operating correctly. In addition to retinal damage, exposure to continuous lighting could have effects on the PK of a test compound. Continuous lighting tends to equalize the day and night food consumption and speed of gastrointestinal (GI) transit rate (Siegel, 1961; Wong and Oace, 1981; Zucker, 1971). Since gastric emptying can be affected by the amount of food in the stomach, the speed of absorption, and hence blood levels, can be changed especially in dietary studies. In addition, continuous lighting may affect the activity levels of the animals and thereby reduce or increase metabolism.

Ventilation

The purpose of an adequate ventilation system is to provide fresh air, control temperature and humidity, remove or dilute chemical pollutants such as ammonia from animal waste, and help to control transmission of infectious agents. Current guidelines recommend a ventilation rate of 10–15 air changes per hour (ILAR, 1997). In addition, if recycled air is used within the facility, it must be mixed with at least 50% fresh air for every air exchange, the recycled air must be returned to the room or area from which it was generated, and the preparation of recycled air is sufficient to minimize toxic gases and odors during husbandry practices (Guide for the Care and Use of Laboratory Animals, 1997).

When placing racks in the room, the special arrangement should be considered to reduce the possibility of cross contamination. This is especially important for dietary studies where the food and test article may be aerosolized. It is recommended that racks be placed at least 2 m apart to minimize the possibility for cross contamination and the spread of microorganisms. As with light exposure, ventilation may be different based upon the position on the cage rack. Randomization of dose groups throughout the cage rack and periodic rotation of cage will help to control for varied exposure.

The ideal arrangement for proper ventilation of an animal room is a colony facility that is positive to an anteroom that is negative to both the animal room and the hallway. This will prevent the spread of disease between colonies and reduce the possibility of exposure to test compounds administered in the diet and control odors. If the facility does not allow for this type of design, the animal room can be either positive or negative to the hallway depending upon the purpose of the study. In clean/dirty corridor systems, air should flow from the access corridor (clean), through the animal room, to the dirty corridor (Sontag et al., 1976).

Noise

The effects of noise on the laboratory rat have been extensively researched. Exposure to various noise levels has been associated with the development of a variety of abnormalities and stress indicators. Changes in uterus and adrenal weights as well as reproductive disturbances have been observed (Geber, 1973; Nayfield and Besch, 1981; Sackler et al., 1959). In addition, the noise associated with normal husbandry activities has been associated with marked increase in plasma corticosterone levels (Barrett and Stockham, 1963). Noise within the animal rooms may be unavoidable; current guidelines suggest that noise levels should be maintained below 85 dB whenever possible. Taking this into consideration, animal care staff should be trained to reduce noise as much as possible, and rodents should be housed in facilities separate for large animal species such as dogs or nonhuman primate.

Caging

Different types of caging have been developed for the housing of rodents that take into account the requirements for temperature and humidity, ventilation, lighting, noise, and the health and well-being of the animal. The type of caging used may create a different microenvironment within the cage than that found in the colony room (macroenvironment). When designing a study, the type of caging to be used should be taken into consideration. For rodent, two main types of caging are available. These are the shoebox or solid bottom cage and the hanging or wire mesh cage.

Shoebox Caging

The use of shoebox caging has several benefits when conducting toxicology studies. The use of solid bottom caging may reduce the incidence of foot lesion in chronic and carcinogenicity studies (Peace et al., 2001). The Association for the Assessment and Accreditation of Laboratory Animal Care has strongly recommended that solid bottom caging be used for all rodent studies unless justification for the use of other types of caging can be provided. Shoebox cages are either rectangular or square boxes with solid bottoms and walls. They may be made of stainless steel, but molded plastics such as polycarbonate (clear) or polypropylene (translucent) are more common (Figure 3.1). Lids for this type of cages are made of wire mesh capable of holding both feed and a water bottle, metal with ventilation holes, or filter tops. Cages may also be suspended from shelves in a rack, in which case the shelf acts as the cover for the cage.

Traditional filter top lids provide some protection from airborne contaminants (Brick et al., 1969; Kraft, 1958; Simmons and Brick, 1970). A disadvantage of this type of caging is that ventilation
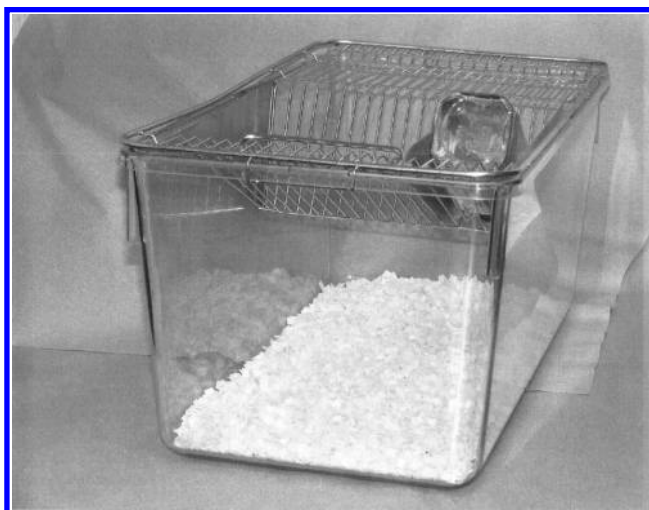
Figure 3.1   Solid bottom shoe box cage.

within the cage is reduced leading to increased temperature, humidity, and levels of carbon dioxide and ammonia (Gordon and Fogelson, 1994; Serrano, 1971; Simmons et al., 1968). This has led to the development of caging that is entirely enclosed. The animal cage in conjunction with the cage rack creates a microenvironment suitable for the rat. The rack system has a fresh air supply that may be HEPA filtered and an air return system that removes the air and filters for contaminants (Figure 3.2). This type of caging also has the added benefit of reduced husbandry. Due to the constant airflow in

Figure 3.2   Rack of suspended wire mesh cages.

the cage, urine and feces are quickly dried and ammonia is removed; as a result, bedding does not require changing as often (Kanzaki et al., 2001).

Typical shoebox caging has sufficient floor space for up to three adult rats group housed or a singly housed dam with litter. The cage may be designed to accept a water bottle or may have openings in one end that allow the cage to be placed on a rack with an autowatering system. Animals may have access to feed via feed jars placed in the cage or via a specially designed lid that has a bin for holding block diet. Shoebox cages can also be designed with a rotating feed system that allows for controlled administration of fixed amounts of diet. Recent studies have shown that controlled or restricted feeding in long-term studies may decrease the incidence of some tumors and may increase survival rates in carcinogenicity studies (Clinton et al., 1997; Toriyama-Baba et al., 2001). The floor of the shoebox cage is usually filled with absorbent bedding. The choice of bedding should be made with care. The use of cedar or soft wood bedding should be avoided as they have been associated with increases in adverse events such as hepatic microsomal enzyme levels (Weichbrod et al., 1988) and increased pup mortality (Burkhart and Robinson, 1978).

Shoebox cages also have several disadvantages when used in rat toxicology studies. Rats are coprophagy and may receive additional exposure to the test compound or its metabolites that have been excreted in the feces. This is of particular importance when evaluating PK data or metabolite profiles. Rats also have a tendency to eat the bedding that may reside in the stomach or impede gavage dosing. In dietary studies, bedding may serve as an alternate food source if the test article alters feed palatability. Due to these factors, alternate caging types may be considered.

Suspended Caging

Suspended wire bottom cages are typically made entirely of metal. The wire mesh floor is designed to allow excrement to fall through to absorbent paper or bedding placed under the cage. This design reduces the need to disturb the animal during routine husbandry. In addition, this type of caging reduces the possibility of increased drug exposure through the consumption of excrement and eliminates the risk of an impeded or obstructed stomach or esophagus during gavage dosing. This cage design also allows for increased ventilation in the cage, thereby reducing ammonia levels.

However, this cage design offers little protection against airborne transmission of disease or contaminants such as test compound in diet admixture studies (Sansone et al., 1977). Wire mesh floor cages have also been associated with a higher incidence of decubital ulcers in studies greater than 1 year in duration (Peace et al., 2001).

When designing a study, the type of caging to be used should be chosen carefully. Both solid bottom and wire mesh cages have advantages and disadvantages. For short-term subchronic toxicology studies, wire mesh cages may be appropriate as it reduces the risk of increase drug exposure. For chronic and carcinogenicity studies, solid bottom or a combination of solid bottom and wire mesh cages may be used.

Minimum Space Recommendations

Current minimum space recommendations for the rat are given in Table 3.4. When deciding how many rats to house per cage, it must be remembered that the population density can affect heat, moisture, oxygen, carbon dioxide, and ammonia in the cage (Clough, 1976). Several studies have been done comparing the effects of group versus individual housing. Overcrowding has been shown to increase plasma corticosterone levels (Barrett and Stockham, 1963). Rats individually housed for 3 months develop larger adrenal and thyroid glands and smaller spleens and thymus glands and display an increased sensitivity to the cardiotoxicity of isoprenaline (Balazs et al., 1962; Dairman and Balazs, 1970). The rat is a social animal and whenever possible should be group housed in pairs or groups of three. For the purpose for most toxicity studies, this may not be practical, but should be considered.

Table 3.4   Minimum Cage Sizes for Rats

| | Floor Area/Animal[a] | |
Weight (g)	in²	cm²
Up to 100	17	110
101–200	23	150
201–300	29	190
301–400	40	260
401–500	60	390
Over 500	70	460

Source: Courtesy of ILAR (1997).
[a] Cage height should be at least 7 in. (17.78 cm).

Food

Physical Form and Presentation

Feed is typically supplied as either pellets or meal to rats. Pellets are usually presented to rats in a metal hopper attached to the front of the cage for suspended wire mesh cages and in wire bins in the lid of shoebox cages. Pelleted diets are considered a more efficient form in that they are less likely to be scattered by the animals and are more easily handled by caretakers. Pelleted diets are also made to be relatively hard and require gnawing, which helps to wear down the incisors of the animal. Pelleted diets are available from a variety of commercial manufactures and may be formulated to specific needs. The meal form of diet is useful when test compounds must be mixed with the diet or for conducting food consumption. The feed is presented to the animals in wide-mouthed jars with lids. In addition to the lid, a perforated metal disk can be placed in the jar; this helps to reduce spillage and contamination by excreta (Figure 3.3). Devices have also been developed for pair-feeding studies or for studies where feed aliquots must be presented throughout the day (Loveless et al., 1972; Quarterman et al., 1970). For specific applications, semimoist diets made with powdered ingredients mixed with water (50% v/v) and agar (0.1%) or liquid diets may be used.

Figure 3.3   Example of dietary meal food jar with lid.

Nutrition

Nutrient Requirements

Nutrient requirements for the laboratory rat have been specified by the Nation Research Council (1995). Recommendations for dietary nutrient contents are presented in Table 3.5. Several complete rat diets adequate for normal growth, reproduction, and maintenance are available from commercial sources. These diets are available in natural ingredients and purified forms. Natural ingredient diets use unrefined or minimally processed ingredients such as cereals, cereal bioproducts, fish meals, soya bean meals, skimmed milk, meat and bone meals, and molasses (Clarke et al., 1977). Purified diets use refined ingredients such as casein, vegetable oil, starch, and sucrose. Cellulose is used as a nonnutritive filler in purified diets.

Natural ingredient diets are more economical and more widely used; however, the variability of the nutrient content may make interpretation of toxicology results difficult. For the purpose of toxicology studies conducted under good laboratory practices (GLPs), diets should be purchased from a vendor that certifies the diet. A certificate of analysis should be obtained for each lot of feed purchased (Environmetal Protection Agency, 1979; Food and Drug Administration, 1978; ILAR, 1997; National Academy of Sciences, 1978).

Food Restriction in Chronic Studies

Food restriction in chronic studies has been found to increase life expectancy and decrease the incidence of some types of tumors. Keenan et al. (1998) found that a 25% and 50% restriction of food consumption resulted in decreased benign and malignant tumor incidence and increased survival rates (Table 3.6). In addition, animals placed on feed restriction were found to have lower overall body weights, which can be correlated to the incidence of plantar lesions. When planning a chronic toxicity or carcinogenicity study, consideration should be given to placing animal on feed restriction.

Sterilization

Diet sterilization is mandatory for gnotobiotic animals or immunocompromised animals and is recommended for diets used for specific pathogen-free (SPF) animal (Coates, 1984; Wostman, 1975). Common means of sterilization are heat, ionizing radiation, and fumigation. Heat may affect the nutrient value of diets and the physical nature of the diet by causing the pellets to disintegrate or become hard or increase the tendency for mold growth. Fumigation with ethylene oxide has proven effective, but may change the nutrient value, and care must be given to ensure all traces of ethylene oxide have been removed. The use of ionizing radiation has proven to be the most effective means of sterilization without affecting the nutrient value.

Water

Bottle or automatic watering systems are used to supply water to the animals. Where bottles are used, they are capped with rubber stoppers fitted with stainless steel tubes. The tubes have constricted apertures or ball valves. When the bottles are inverted and attached to the cages, rats can lick the aperture or valve and a drop of water will be delivered. Water bottle systems are useful when water consumption is to be measured or the test compound is to be delivered in the water. This is inexpensive to set up, but incurs increased labor costs due to bottle washing, sterilization, and replacement required; bottles are commonly changed every 2–3 days.

Table 3.5   Recommended Dietary Content of Nutrients for Rats

Nutrient	Amount in Diet (90% Dry Matter)[a]	Amount in Diet (PMI Certified Diet)[b]
Protein (%)	15.0	20.1
Fat (%)	5.0	5.1
Digestible energy (kcal/kg)	3800	4040
L-amino acids		
Arginine (%)	0.6	1.13
Asparagines (%)	0.4	
Glutamic acid (%)	4.0	4.2
Histidine (%)	0.3	0.49
Isoleucine (%)	0.5	1.03
Leucine (%)	0.75	1.58
Lysine (%)	0.7	1.18
Methionine (%)	0.6	0.43
Phenylalanine (and tyrosine) (%)	0.8	0.88
Proline (%)	0.4	1.47
Threonine (%)	0.5	0.78
Tryptophan (%)	0.15	0.24
Valine (%)	0.6	1.05
Nonessential (%)	0.59	
Minerals		
Calcium (%)	0.50	0.8
Chloride (%)	0.005	0.47
Chromium (mg/kg)	0.3	2.0 (ppm)
Copper (mg/kg)	5.0	11 (ppm)
Fluoride (mg/kg)	1.0	13 (ppm)
Iodine (mg/kg)	0.15	0.77 (ppm)
Iron (mg/kg)	35.0	210 (ppm)
Magnesium (%)	0.05	0.21
Manganese (mg/kg)	10.0	75 (ppm)
Phosphorus (%)	0.30	0.60
Potassium (%)	0.36	0.86
Selenium (mg/kg)	0.15	0.25 (ppm)
Sodium (%)	0.005	0.30
Sulfur (%)	0.03	0.25
Zinc (mg/kg)	12.0	76 (ppm)
Vitamins		
A (IU/kg)	2300	18000
D_3 (IU/kg)	1000	2200
E (IU/kg)	27.0	66
K_1 (mg/kg)	1.0	0.4 (ppm)
Choline (mg/kg)	750	1800 (ppm)
Folic acid (mg/kg)	1.0	4.0 (ppm)
Niacin (mg/kg)	15.0	95 (ppm)
Pantothenate (calcium) (mg/kg)	8.8	17 (ppm)
Riboflavin (mg/kg)	3.0	8.0 (ppm)
Thiamin (mg/kg)	4.0	16 (ppm)
Vitamin B_6 (mg/kg)	6.0	6.0 (ppm)
Vitamin B_{12} (mg/kg)	0.05	0.02

[a]   Values taken or calculated from National Research Council (1995).
[b]   PMI® Nutrition International.

Table 3.6   Effects of Feed Restriction in Chronic Toxicity Studies

Parameter	Ad Libitum		25% Restriction		50% Restriction	
	M	F	M	F	M	F
Body weight (g)	765 ± 145	611 ± 87	572 ± 49	296 ± 22	357 ± 24	216 ± 15
Tumor incidence (%)	78	94	78	87	58	54
Benign tumor (%)	70	94	66	82	46	50
Malignant tumor (%)	26	30	22	40	16	10
Survival (%)	18	18	68	56	78	82

Source: Keenan, K. P. et al., *J. Toxicol. Environ. Health Part B*, 1, 101, 1998.

Autonomic watering systems connect the cage and rack to the laboratory water supply by a series of pipes and tubes. Animals activate the system by licking a valve attached to the cage. The system requires less maintenance, but can malfunction if air becomes trapped in the rack, laboratory water pressure fails, or the rack is incorrectly attached to the laboratory plumbing. Since cages and racks are connected by plumbing, there is an increased risk of pathogen spread. Microorganism buildup in the plumbing is minimized by flushing off the system. In both systems, a malfunction could result in flooding of shoebox cages, and animals should be monitored periodically each day.

Since the water supply can be a source of environmental contamination, current toxicology guidelines and regulation require periodic analysis of water for contaminants that may affect the outcome of the study.

Prevention of Infectious Diseases

The result of a toxicology study will be affected by a disease outbreak, which results in the death or severe clinical illness of the test animals. Less pathogenic agent causing subclinical or less severe signs may also affect study results by suppressing or modifying the immune response, affecting fetal viability or organogenesis, or causing specific histological changes (Hsu et al., 1980; Jacoby and Barthold, 1981). By suppressing immune response, a mycoplasma infection can result in an increase in the incidence of lung tumors (Schreiber et al., 1972). If only a portion of the animals on study are affected, this could lead to misleading or uninterpretable results. It is recommended for subchronic toxicity greater than 90 days in length, chronic toxicity, and carcinogenicity studies; sentinel animals should be placed in the room. On a weekly basis, a small amount of soiled bedding from other colony animals is transferred to the sentinel animal cage. This exposes the animals to microbial status of other study animals. Periodically throughout the course of the study, these animals should be submitted for clinical pathology and necropsy evaluation. This evaluation will help to monitor the health status of the study animals over the course of the study.

Microbial Status

As study results may be affected by infectious disease, researchers have moved toward using animals of known health status. The range of different levels of microbic association goes from "axenic" (or germ-free) animals, which are produced by hysterectomy and reared behind germ-free barriers, to conventional rats from which microbes have not been excluded. SPF or "barrier-reared" (BR) rats are animals derived from axenic animals, reared in barrier-protected colonies, and shown to be free of certain pathogens. The SPF animal is preferred by researcher, especially for longer-term studies.

Disease Prevention

One challenge facing the toxicologist and laboratory management is to maintain the microbial and health status of the animals once they arrive at the facility.

It is recommended to purchase animals from a vendor that breeds animals for the purpose of research. These vendors will typically ship animals in groups in boxes with polyester fiber filter that helps to avoid contamination of the animals in transit. This is especially important if using immunocompromised animals. In order to minimize the stress animals face during transportation to the laboratory, the vendor should provide sufficient feed and water and, if possible, ship the animals in environmentally controlled vehicle. To avoid contamination from the outside of the shipping containers, these should not be brought into the room where animals will be quarantined.

Once received at the facility, animals should be quarantined to allow them to recover from the stresses of transit, resume normal weight gain, and allow existing disease to express itself. The period recommended for quarantine varies from 48 hours to 4 weeks, but a period of 2 weeks is typical for most facilities. Animals may be quarantined in a specially designed room or in the room to be used for the study. For longer-term studies, baseline values for a disease-surveillance program can be established during this period: groups of excess animals obtained for this purpose can be euthanized and examined upon receipt and at preselected intervals during the course of the study for various serological, clinical laboratory, and histological examination.

Elements in barriers against disease introduction include (1) how materials, including equipment, feed, and bedding, will be handled as they are brought into the room, (2) what are the personnel entry procedures, and (3) how is the environmental system handled. ILAR (1997) discusses procedure to be used for various classifications of barrier areas. Specific procedures will be determined by the research objectives, the health of the animals, and the cost of repeating a compromised study.

Jacoby and Barthold (1981) discuss various options available to the investigator if an outbreak occurs. These include ignoring the infection, quarantine of specific animal or colony, purposeful exposure of animals to generalize the infection, culling, and termination of the study or population. The selection of an option will be dependent on the type of infection and chances for control, continued health of the animals, research objectives, husbandry practices, and facility design. It is strongly recommended that if an outbreak occurs, the researcher should consult with the attending veterinarian to determine the best course of action to take.

Study Design

The length and design of toxicology studies used to predict human risk are governed by guidelines issued by regulatory bodies such as the U.S. Food and Drug Administration (FDA), the International Council on Harmonization (ICH), the Environmental Protection Agency (EPA), and their counterparts worldwide. Toxicology studies are divided into a series of three sets of studies that are required for each phase of clinical trials. For initial approval to begin clinical trails, the following studies are required. The length of dosing in the toxicology studies varies depending upon the intended length in clinical trails. A test compound intended to be a repeat dose study for up to 28 days in duration initially requires a two-phase study in which a maximum tolerated dose (MTD) following a single administration is determined followed by a second phase during which the test compound is administered daily at dose levels based upon the MTD for 5–7 days (Table 3.7). Following the completion of the MTD study, a 14- or 28-day repeat dose study should be conducted (Tables 3.8 and 3.9). These studies assess the effects of a test compound at dosages that do not cause immediate toxic effects.

Table 3.7   MTD Study in Rats

Phase A	Oral MTD Study	
	Males	Females
Dose level 1	3	3
Dose level 2	3	3
Dose level 3	3	3
Dose level 4	3	3

	7-Day Oral Range–Finding Study			
	Main Study		Toxicokinetics	
Phase B	Males	Females	Males	Females
Control	5	5	—	—
Low dose	5	5	9	9
Mid dose	5	5	9	9
High dose	5	5	9	9

Experimental design: In Phase A, the dose level will be increased until the MTD is determined. The MTD is a dose that produces neither mortality nor more than a 10% decrement in body weight nor clinical signs of toxicity. In Phase B, animals will be dosed daily for 7 days at fractions of the single dose MTD to estimate a repeat dose MTD.

Dose route/frequency: As requested.

Phase A: Once/Phase B, once per day for 7 consecutive days.

Observations: Twice daily in both phases (mortality/moribundity).

Detailed clinical observations: Daily in both phases.

Body weights: Daily in both phases.

Food consumption: Daily.

Clinical pathology (Phase B only): Hematology, clinical chemistry, and urinalysis evaluations on all surviving main study animals at termination.

Necropsy (Phase B only): Tissues saved for possible future histopathological evaluation.

Organ weights (Phase B only): Adrenals, brain, heart, kidneys, liver, lungs, ovaries with oviducts, pituitary, prostate, salivary glands, seminal vesicles, spleen, thyroid with parathyroid, thymus, testes, uterus.

Toxicokinetics: Blood collected on Days 1 and 7 (3 cohorts consisting of 3 animals/sex/treatment group bled twice to equal six time points), calculation of C_{max}, T_{max}, AUC_{0-24}, and $T_{1/2}$.

In support of Phase 2 clinical trails, longer-term subchronic and chronic toxicity studies (Table 3.10) should be conducted. Subchronic and chronic toxicity studies are designed to assess the test compound effects following prolonged periods of exposure. The highest dosage level in each of these studies should produce a toxic effect such that target organ may be identified. The lowest dosage level should provide a margin of safety that exceeds the human clinical dose and ideally allows for the definition of a no observable effect level. Alternatively, when effects relate to the pharmacological mechanism of the test compound or when observed effects may be related to treatment with the test compound but may not be of toxicological significance, a no observable adverse effect level may be determined.

In addition to the subchronic and chronic toxicity studies in support of Phase 2 clinical trials, reproductive safety studies may also be required. Reproductive toxicity studies are typically required for test compound intended to be administered to women of childbearing age or may affect male reproduction. These studies include an assessment of the potential effects of the test compound on general fertility and reproductive performance (Segment I), developmental toxicity (Segment II), or perinatal and postnatal development (Segment III). The highest dose in reproductive studies should be chosen so that administration causes some minimal toxicity. Typically, a dose range–finding pilot study in a small number of animals should be conducted prior to initiating the definitive reproductive toxicology studies. Examples of protocols designed to meet the ICH guidelines are presented in Tables 3.11 through 3.13.

Table 3.8   14- or 28-Day Repeat Dose Toxicity Study in Rats

	Main Study*		Toxicokinetics	
	Males	**Females**	**Males**	**Females**
Vehicle control	10	10	—	—
Low dose	10	10	9+3[a]	9+3[a]
Mid dose	10	10	9+3[a]	9+3[a]
High dose	10	10	9+3[a]	9+3[a]

[a] Three additional animals/sex/treatment group included as replacement animals.
Dose route/frequency: As requested.
Observations: Twice daily (mortality/moribundity).
Detailed clinical observation: Weekly.
FOB: Pretest and Day 14 or 25.
Body weights: Weekly.
Food consumption: Weekly.
Ophthalmology: All animals prior to test article administration; all surviving main study animals at study termination.
Clinical pathology: Hematology, clinical chemistry, and urinalysis evaluations on all surviving main study animals at termination.
Toxicokinetics: Blood collected on Days 1 and 14 or 27 (3 cohorts consisting of 3 animals/sex/treatment group bled twice to equal six time points); TK modeling.
Necropsy: All main study animals; toxicokinetic animals euthanized and discarded.
Organ weights: Adrenals, brain, heart, kidneys, liver, lungs, ovaries with oviducts, pituitary, prostate, salivary glands, seminal vesicles, spleen, thyroid with parathyroid, thymus, testes, uterus.
Slide preparation/microscopic pathology: All animals in the vehicle control and high-dose groups and all found dead animals; full set of standard tissues; low- and mid-dose group target organs (to be determined); gross lesions from all animals.

Table 3.9   28-Day Repeat Dose Toxicity Study with Immunophenotyping in Rats

	Main Study		Toxicokinetics	
	Males	**Females**	**Males**	**Females**
Vehicle control	10	10		
Low dose	10	10	9+3[a]	9+3[a]
Mid dose	10	10	9+3[a]	9+3[a]
High dose	10	10	9+3[a]	9+3[a]

[a] Three additional animals/sex/treatment groups included as replacement animals; the control animals will not be evaluated for toxicokinetics.
Dose route/frequency: As requested/once daily all animals for 28 days.
Observations: Twice daily (mortality/moribundity).
Detailed clinical observation: Weekly.
FOB: Pretest and Day 25.
Body weights: Weekly.
Food consumption: Weekly.
Ophthalmology: All animals prior to test article administration; all surviving main study animals at study termination.
Clinical pathology: Hematology, clinical chemistry, and urinalysis evaluations on all surviving main study animals at termination.
Immunotoxicology: Immunophenotyping of blood leukocytes by flow cytometry on all surviving main study animals at termination. NK cell assay on blood leukocytes of all surviving main study animals at termination.
Toxicokinetics: Blood collected on Days 1 and 27 (three cohorts consisting of three animals/sex/treatment group bled twice to equal six time points).
Necropsy: All main study animals; toxicokinetic animals euthanized and discarded.
Organ weights: Adrenals, brain, heart, kidneys, liver, lungs, ovaries with oviducts, pituitary, prostate, salivary glands, seminal vesicles, spleen, thyroid with parathyroid, thymus, testes, uterus, two lymph nodes (e.g., mesenteric, axillary, popliteal, etc.) including the lymph node draining the route of administration.
Slide preparation/microscopic pathology: All animals in the vehicle control and high-dose groups and all found dead animals; full set of standard tissues (add Peyer's patch, extra lymph node); low- and mid-dose group target organs; gross lesions from all animals.

Table 3.10   Subchronic and Chronic Toxicity Study in Rats

	Main Study		Toxicokinetics	
	Males	**Females**	**Males**	**Females**
Vehicle control	15	15	—	—
Low dose	15	15	9+3[a]	9+3[a]
Mid dose	15	15	9+3[a]	9+3[a]
High dose	15	15	9+3[a]	9+3[a]

[a] Three additional animals/sex/treatment group included as replacement animals.
Dose route/frequency: As requested.
Observations: Twice daily (mortality/moribundity).
Detailed clinical observation: Weekly.
Body weights: Weekly.
Food consumption: Weekly.
Ophthalmology: All animals prior to test article administration; all surviving main study animals at study termination.
Clinical pathology: Hematology, clinical chemistry, and urinalysis evaluations on all surviving main study animals at termination.
Toxicokinetics: Blood collected on Days 1 and 90 (three cohorts consisting of three animals/sex/treatment group bled twice to equal six time points); TK modeling.
Necropsy: All main study animals; toxicokinetic animals euthanized and discarded.
Organ weights: Adrenals, brain, heart, kidneys, liver, lungs, ovaries with oviducts, pituitary, prostate, salivary glands, seminal vesicles, spleen, thyroid with parathyroid, thymus, testes, uterus.
Slide preparation/microscopic pathology: All animals in the vehicle control and high-dose groups and all found dead animals; full set of standard tissues; low- and mid-dose group target organs; gross lesions from all animals.

Table 3.11   Study of Fertility and Early Embryonic Development to Implantation in Rats

	Males	**Females**
Vehicle control	25	25
Low dose	25	25
Mid dose	25	25
High dose	25	25

Dose route/frequency: As requested/males dosed beginning 28 days before mating and continuing until euthanasia. Females dosed beginning 14 days before mating and continuing through Day 7 of gestation (implantation).
Observations: Twice daily (mortality/moribundity).
Clinical examinations: Observations for clinical signs, body weights, and food consumption measurements recorded during the study period. Beginning at initiation of test article administration, females examined daily to establish estrous cycle.
Uterine examinations: Performed on dams on Day 13 of gestation. Gravid uterine weight and the weight of the ovaries recorded. Total number of corpora lutea and implantations, location of resorptions, and embryos recorded. Females subjected to necropsy and reproductive organs and gross lesions fixed for possible microscopic evaluation.
Evaluation of males: Following disposition of females, the males euthanized and subjected to necropsy. The testes and epididymides weighed, and analysis of sperm parameters (concentration, motility, and morphology) performed. Reproductive organs and gross lesions fixed for possible microscopic evaluation.
Statistical analysis: Standard.

In support of Phase 3 clinical trials, a carcinogenicity study may be required (Table 3.14). Typically 18 months to 2 years in duration, this type of study is designed to assess the potential of the test compound to induce neoplastic lesions. The highest dosage in a carcinogenicity study should cause minimal toxicity when administered via the intended route for clinical use. The preclinical studies required in support of the clinical trials are dependent upon the intended route and frequency of administration of the test compound and the intended age group to be treated.

Table 3.12 Embryo–Fetal Development in Rats

	Time-Mated Females
Vehicle control	25
Low dose	25
Mid dose	25
High dose	25

Dose route/frequency: As requested/dosing will initiate on Day 6 of gestation and continue to and include Day 17 of gestation.

Observations: Twice daily (mortality/moribundity).

Clinical examinations: Daily gestation days (GDs) 6 through 20.

Body weights/food consumption: GDs 0, 6, 9, 12, 15, 18, and 20.

Cesarean section/necropsy: Litters will be delivered by cesarean section on Day 20 of gestation. Gravid uterine weight will be recorded. Total number of corpora lutea, implantations, early and late resorptions, live and dead fetuses, and sex and individual body weights of fetuses will be recorded. External abnormalities of fetuses will be recorded. Approximately one-half of the fetuses will be processed for visceral abnormalities, and the remaining fetuses will be processed for skeletal abnormalities. All fetuses will be examined for visceral and skeletal abnormalities. Dams will be subjected to necropsy and gross lesions and target organs (if known) will be saved.

Table 3.13 Pre- and Postnatal Development, Including Maternal Function in Rats

	P Generation		F1 Generation	
	Males	Females	Males	Females
Vehicle control	NA	25	25	25
Low dose	NA	25	25	25
Mid dose	NA	25	25	25
High dose	NA	25	25	25

Number on study: P generation, 100 females; F_1 generation, 100 males, 100 females.

Dose route/frequency: As requested/once daily to P animals from GD 6 to postnatal day (PND) 21. F1 animals not dosed.

Observations: Twice daily (mortality/moribundity).

Clinical observations: P females, daily during treatment/F1 adults, weekly.

Body weights:

  P females, GD 0, 6, 10, 14, 17, and 20; PND 0, 7, 10, 14, and 21.

  F1 males, weekly through termination.

  F1 females, weekly until evidence of copulation detected and then GD 0, 7, 10, and 13.

Food consumption: P females, on corresponding body weight days during gestation/lactation.

Vaginal smears: All F1 females during a 21-day cohabitation period until evidence of copulation is detected.

Litter evaluations: All F1 offspring, count, body weight, sex, clinical observations on PND 0, 4, 7, 14, 21; behavioral and developmental evaluation of four males and four females from each litter for static righting, pinna detachment, cliff aversion, eye opening, air drop righting reflex, neuropharmacological evaluation, auditory response. One male and one female (selected for the next generation) tested for sexual maturation (vaginal opening, preputial separation), motor activity/emotionality, and passive avoidance.

Cesarean section: On GD 13, F1 females for location of viable and nonviable embryos, early and late resorptions, number of total implantations, and corpora lutea.

Sperm evaluation: May be conducted on F1 males if evidence of reduced fertility is noted (additional cost).

Necropsy: Gross lesions/target organs fixed for possible microscopic evaluation (additional cost).

All P females at PND 22 as well as all F1 weanlings not selected for F1 generation.

All F1 females at GD 13.

All F1 males after termination of F1 cesarean sections.

Table 3.14 Carcinogenicity Study in Rats

	Main Study		6-Month Satellite	
	Males	**Females**	**Males**	**Females**
Vehicle control	60	60	20	20
Low dose	60	60	20	20
Mid dose	60	60	20	20
High dose	60	60	20	20

Dose route/frequency: As requested.
Observations: Twice daily (mortality/moribundity).
Detailed clinical observations: Once weekly.
Body weights: Weekly for first 13 weeks, monthly thereafter.
Food consumption: Weekly for first 13 weeks, monthly thereafter.
Ophthalmology: All animals pretest and all survivors prior to terminal sacrifice.
Clinical pathology:
Main study: Hematology at termination.
6-month satellite: Hematology, clinical chemistry, and urinalysis evaluations on all
    surviving satellite animals at termination.
Necropsy: All animals.
Slide preparation/microscopic pathology: All animals, full set of standard tissues, all
    masses and all lesions.
Statistical analysis: Standard.

Dosing Techniques

A wide range of dosing techniques have been developed for evaluating the effects of test compounds in the rat. Due to the requirement in toxicology studies to extrapolate results to human risk, convention and guidelines dictate that rats should be dosed by a route closely approximating that of human exposure. For mechanistic studies, where specific organs may be targeted, the route of administration will be dictated by the research objectives.

Oral Route

Rodents have several unique characteristics to be considered regarding the oral administration of test compounds. One of the most important characteristics is the lack of an emetic response. The lack of this response allows for a higher dose of a potential emetic compound to be administered and evaluated. Many compound and excipients may cause emesis in dogs or other large animal species and may lead to a low level of exposure and erratic blood levels. A second factor to consider is that rodents are nocturnal and eat most of their food at night. When maintained on a 12-hour light dark cycle, rats have been found to consume 75% of their daily food intake during the dark cycle (Wong and Oace, 1981). This should be taken into consideration when designing an oral gavage study and determining when the animal may be dosed. Early in the light cycle, animals are more likely to have a full stomach, and complications associated with dosing may occur if large volumes of test article are administered. In addition, a full stomach may affect gastric emptying and the rate of absorption of an orally administered test compound.

Techniques for oral administration of test compounds include mixing in the diet, via gavage or stomach tube, via capsule, or via drinking water. The most widely used method of oral administration is the dietary and gavage techniques.

Dietary versus Gavage Methods

The choice between dietary and gavage dosing techniques is typically based upon several factors. A scientific decision can only be made with a knowledge of the PK of the test compound administered by both methods. Other considerations that may be used in making this decision are as follows.

The dietary method that can be used is a compound that can be mixed with the diet, is stable under storage conditions in the diet, and is palatable to the animal. A major advantage of the dietary method is that it requires less manpower to perform the study. The diet mixing process can be performed weekly or, if stability allows, less often. The mixing and feeding process is less labor intensive than gavaging rats on a daily basis. Several disadvantages also exist in using the dietary method. Methods must be developed and validated to prove homogeneity and stability. This is not as easy a process as with a suspension or solution. The dietary method is also less exact than the gavage method, in that the concentration of compound mixed in the feed is based upon predicted feed consumption and body weights. In addition, if the feed is not palatable to the animal, or the test compound makes the animal ill, feed consumption may be reduced, thereby reducing exposure to the test compound. In addition, the facility and control animals may be exposed to the test compound through dust or vapors.

The gavage method may be used when the test compound is not stable in the diet or may not be palatable to the animals. In addition, the gavage method is preferable when evaluating toxicokinetics or PK. As with dietary mixtures, test compound administered via gavage as a solution or suspension should be analyzed for homogeneity, stability, and concentration. Methods for solution or suspension may be easier to develop than those required for dietary mixtures. For GLP studies, evaluation of homogeneity, stability, and concentration should be conducted for every study. If the same methodology and batch size are used for multiple studies, homogeneity may be established one time. Stability of the test compound in solution or suspension should be determined under the testing conditions in the proposed vehicle. Typically, stability for toxicology studies is established for between 7 and 14 days. If the test compound is not found to be stable, stability of shorter duration may be established. Lastly, concentration analysis should be established for each dose level and should be periodically evaluated during longer-term studies.

With the gavage method of dosing, a more precise amount of the test compound can be delivered and may reduce the amount of test compound required to complete the study. This becomes important when evaluating the effects of a pharmaceutical, as the required dose levels and exposure levels to show safety may be lower than that required for a pesticide or chemical. A disadvantage of the gavage method is that it involves handling of the rat for each dosing. Handling of the rat has been shown to increase corticosterone levels (Barrett and Stockham, 1963) and may affect study results. Additionally, daily intubation may lead to death due to esophageal puncture or inhalation pneumonia.

Dietary Method

When utilizing the dietary method, the test compound is mixed with the diet and administered to the animals either ad libitum or presented to the animals for a fixed amount of time each day. The dosage received by an animal is regulated by varying the concentration of test compound in the diet based on the predicted food consumption and body weight. Food consumption and body weight predictions are based upon historical laboratory data for early time points in a study. As the study progresses, growth and food consumption curves can be established for each group and group mean data can be used to predict future food consumption. Different concentrations of the test compound and diet should be made for each sex.

Test compounds and diets are mixed in two steps: (1) the compound and about 10% of the total amount of diet are blended in a premix, and then (2) the premix and the remainder of the diet are mixed. The total amount of diet to be mixed is first weighed out; the 10% is separated into the premix. To make the premix, all of the test compounds and an aliquot of the diet (from the 10%) are put into a mortar. These ingredients are ground with a pestle until the mixture appears homogeneous. The mixture and the remainder of the premix are then layered in a small capacity mixer and mixed for 5–10 min. The time for this mixing process can be varied if analysis shows the total mixture is not homogeneous. For the final mix, the premix and the remainder of the diet are layered in a large

capacity mixer. The mixing time will vary with the type of blender and can be varied if the analysis shows that the total mixture is not homogeneous.

Several types of blenders are available for the mixing process; these include open-bowl "kitchen" mixers, V or PK blenders, and Turbula mixers. Metal parts should be ground to eliminate electrostatic forces. Food jars and other equipment for presentation of compound diet mixtures have been previously discussed. In addition, alternative methods of dietary administration such have microencapsulation may be used for volatile, reactive, or unpalatable chemicals.

Gavage Method

In the gavage procedure, the test compound is administered by passing a feeding tube or gavage needle attached to a syringe down the esophagus into the stomach.

Test article preparation: If not already a liquid, the test compound is prepared for administration by adding it to the appropriate vehicle. The choice of vehicle will depend on the characteristics of the compound and whether it is to be administered as a suspension or a solution. In addition, consideration must be given to the effects of the vehicle on the rat (Gad and Chengelis, 1988). Common vehicle used include water and food grade oils such as corn oil. Suspensions are made when aqueous vehicles are desired and the test compound is not soluble. Suspending agents such as methylcellulose are added to increase the viscosity and hold the compound in suspension. Other agents such as Tween 80, ethanol, polyethylene glycol 400, and others may be used as wetting or stabilizing agents.

Equipment: Soft catheters made of silastic or polyethylene (e.g., infant feeding tubes), stainless steel gavage needles with smooth ball-shaped tips, or polyethylene gavage needles with ball-shaped tips are commonly used. All are commercially available and are relatively inexpensive. While the soft catheter minimizes the chance of esophageal trauma, liquid can leak past the catheter and back up the esophagus and be aspirated. The ball-shaped tips of the gavage stainless steel gavage needles reduce the changes of tracheal injections; however, if an animal struggles while the needle is in the esophagus, the rigid needle increases the chances of perforating the esophagus. The polyethylene gavage needle incorporates the best of both the soft catheter and the stainless steel needle, but due to the flexible nature of the needle, the risk for tracheal injection is increased.

Conybeare and Leslie (1988) found that deaths in gavage studies were a result of aspiration of small amounts of irritant solutions or acidic, hypertonic solutions. They also found that the use of a ball tip 4 mm in diameter helped to eliminate deaths related to dosing. With gentle handling, the animals will be acclimated to the techniques used and dosing will become easier.

Aspiration and tracheal administration of test compound as well as esophageal trauma have been associated with gavage dosing and may lead to difficulty in interpretation of the study. The catheter and the needles all have risks inherent in their use; therefore, care should be used when using these tools and animal technician should be properly trained. The choice of the appropriate catheter or needle should be left up to the technician dosing and should be whatever the technician has been trained and is most comfortable with.

Technique: The description in the following is appropriate for either a gavage needle or catheter; for simplicity, only the needle will be mentioned in the description. The method for holding a needle and syringe combination while dosing is illustrated in Figure 3.4; the method for the catheter and syringe combination is shown in Figure 3.5.

Prior to picking up the animal, the syringe should be attached to the needle and filled with the appropriate amount of test compound to be delivered. Any air bubbles should be eliminated and the needle wiped clean of residual test compound. This is done so that the animal does not taste the test compound and residual test compound is not aspirated as the needle is passed down the esophagus. If the dosing liquid is distasteful, the animal may struggle after repeated dosing and increase the chances of being injured.
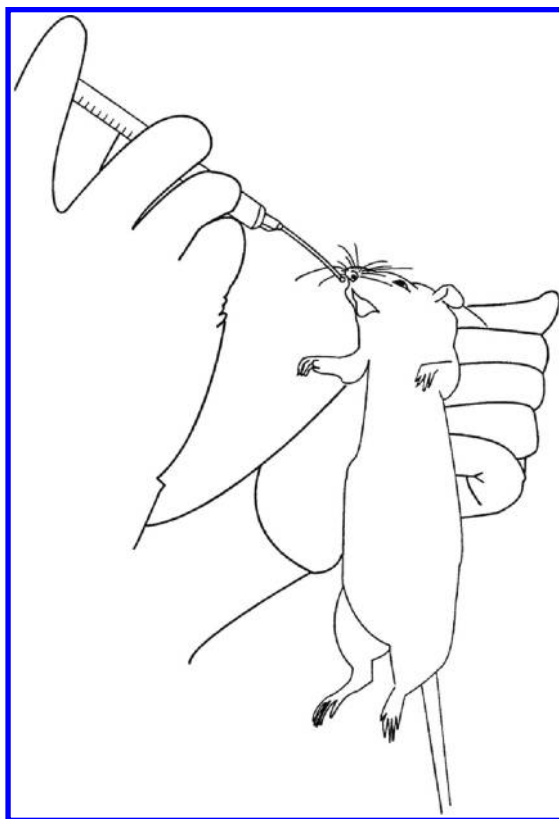
Figure 3.4   Gavage dosing with ball-tipped needle. Note method of restraint.

To position the animals for gavage, it should be grasped by the skin of the back and neck (see Figure 3.4) ensuring that the head, neck, and back are in a straight line. Alternatively, the animals can be grasped about the shoulders, with the index finger and thumb on either side of the head (see Figure 3.5). The objective is to firmly hold the animals to be able to control any struggling if it occurs and to also prevent the animal from biting the technician. For even more control, the animal may be placed on a table or brought up against the operator's chest.

Once the animal is in position, the needle can be inserted into the mouth of the animal and moved over the tongue and down into the esophagus. The length of the needle should be inserted into the animal. A slight rotation of the needle may help with insertion into the esophagus. If the needle is inserted into the trachea, the animal may struggle. The syringe should be grasped lightly such that, if the animal does struggle, the chances of an esophageal tear are minimized. If the animal continues to struggle, the needle should be withdrawn to allow the animal to calm down, and then dosing should be attempted again. Alternatively, if a catheter is used, as the tube is placed into the mouth, it should be placed to the side between the molars. This is done because the tube may by bitten or transected if passed too close to the front teeth.

With the needle in place, the test compound should be slowly expelled into the animal. If administered too rapidly, reflux may occur and the test compound may back up into the esophagus, resulting in an inaccurate dose being given and possible aspiration of the test compound. Once the dose has been delivered, the needle should be withdrawn and the animal observed for any signs of distress or respiratory difficulty. An experienced technician should be able to dose between five and seven animals per minute without causing discomfort to the animals and with minimal dosing-related deaths.
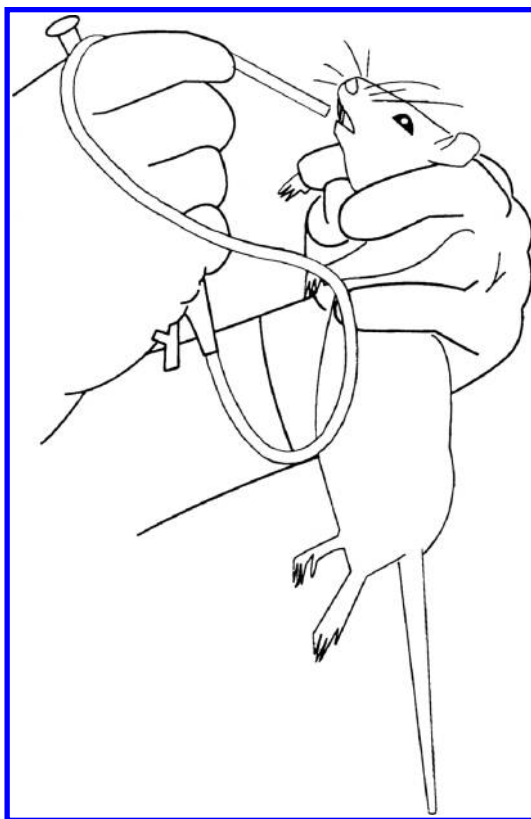
Figure 3.5 Gavage dosing with infant feeding tube. Alternate method of restraint.

Gavage liquids are commonly administered at a volume of 5–10 mL/kg body weight. The volume should be enough to be delivered accurately, but not so much that it will adversely affect the animal. The maximum volume should be no more than 20 mL/kg. If using volumes greater than 10 mL/kg, it may be advisable to fast the animals for several hours prior to dosing. This will ensure that the stomach is empty prior to dosing and able to handle the larger volume. This option should be considered carefully, as fasting can affect the rate of absorption and clearance from the stomach. In addition, the choice of housing and bedding should be considered when dosing with large volume as rats have the tendency to eat the bedding, which may hinder gavage dosing. In addition, the volume chosen can have an effect upon the results of the study, and volumes greater than 10 mL/kg should only be used when issues of solubility and exposure exist. Ferguson (1962) found that a change in dose volume from 5% to 1% of body weight could reduce mortality rate from approximately 95% to 5%, respectively, at equivalent doses.

Neonatal administration: Neonatal intragastric injections can be made orally with thin silicone tubing (Gibson and Becker, 1967; Smith and Kelleher, 1973) or by intragastric injection with a 27-gauge needle through the abdominal wall (Bader and Klinger, 1974; Worth et al., 1963). The oral method using silicone tubing is performed in a similar manner to the previously described method in adult rats. The intragastric injection through the abdominal wall is performed by first locating the stomach in the upper left quadrant of the abdomen and then carefully inserting the needle through the abdominal wall into the stomach taking care that the animal does not move. The syringe should be gently aspirated to ensure proper placement and then the injection completed and the needle withdrawn.

Capsule

To eliminate the possibility of dosing errors and to deal with compounds that cannot be delivered through conventional means, methods have been developed for the administration of capsules into the esophagus of the rat. The test compound may be prefabricated into a small capsule, or the test article may be weighed and placed into commercially available capsules. An individual capsule is then placed into a specially designed cup in the end of a gavage needle, and the needle is then inserted into the esophagus of the rat. The capsule is then pushed out of the cup into the esophagus using either air or a rod inside the needle. The needle is then withdrawn and the capsule moves down into the stomach by peristaltic action. Only small amount of test compound can be administered as a single dose using this method, but multiple capsules can be administered sequentially in the same dosing session.

Water

As an alternative to dietary administration, compounds that are water soluble, palatable to the rat, and stable in water may be administered via drinking water. This method offers similar advantages as adding a test compound to the diet. Additionally, compounds will be more easily mixed, and analyses will be more easily developed than when a compound is in the diet. However, spillage of water makes measurement of the actual dose received difficult.

Intravenous Route

One of the most common methods of administration of test compound is via intravenous injection or infusion. The intravenous route is often the route of choice for compounds that have poor bioavailability via the oral route or have a short half-life. Several issues must be considered when administering a test compound intravenously. The compound must be soluble in an acceptable intravenous vehicle or excipient, must be able to be administered as a solution, and should be sterile or sterile filtered prior to administration. In addition, when designing a study, the PK profile of the test compound administered intravenously should be considered. Study activities such as clinical observations and functional observational battery (FOB) should be planned around the expected time of greatest plasma concentration.

A variety of veins may be used for intravenous injections. These include the lateral tail (caudal), jugular, femoral, saphenous, lateral marginal, dorsal metatarsal, sublingual, and dorsal penile veins. While most of these are superficial, and easily available for injection, several require the use of anesthesia or more than one technician and may be of limited use in repeat dose studies. While anesthesia may be acceptable for acute studies or surgical model, its repeated use may have an effect on the toxicity of a test compound.

Lateral Tail Vein

The lateral tail veins are currently the most widely used for intravenous injections in the rat. The veins are easily visible, especially in young animals, and injections can be performed by one person without the use of anesthesia. The technician performing the function should be well trained, and care should be taken to ensure that the lateral veins are being accessed and not the dorsal or ventral artery of the vein.

Bolus Injection Technique

The animal should be placed in an appropriate restrainer. This typically consists of a solid tube that the animal is placed into head first that has a stop that is placed behind the animal with a hole
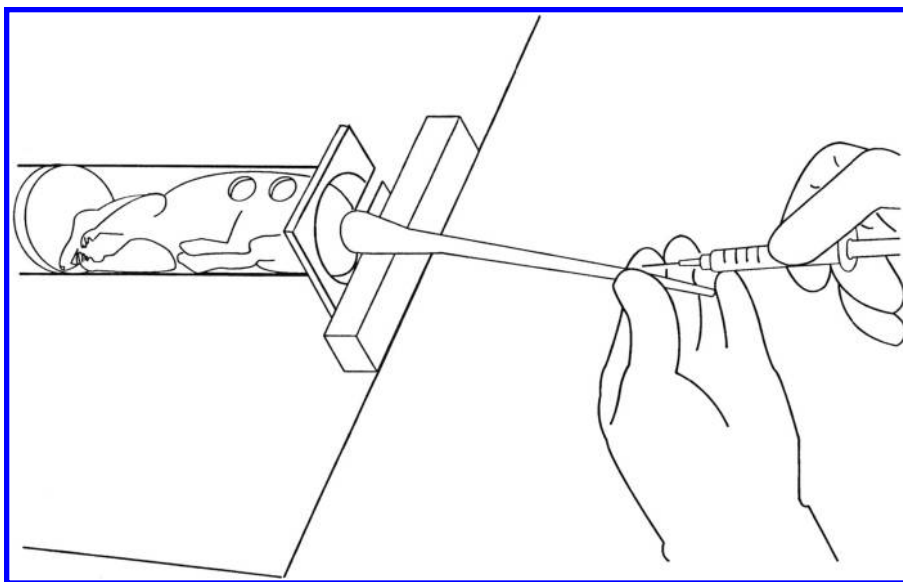
Figure 3.6   Tail vein injection.

that allows the tail to hang out the back (Figure 3.6). The restraint tube is designed to be secure enough that the animal cannot move, back out, or turn, but can still breath comfortably. Once secure, the tail should be cleaned and the vein may be dilated with heat. This may be accomplished by placing the tail in warm water (40°C–45°C), placed under a heat lamp, or wrapped with warm gauze. Care must be taken to avoid using excessive heat as tissue damage may result. Minsaian (1980) describes a tourniquet made from a plastic syringe and thread. If used, this should not be left on for an extended period of time.

When performing an injection, the end of the tail should be held firmly and taut with the thumb and index finger of one hand. A 23-gauge needle attached to an appropriately sized syringe should be held with the bevel up at a shallow angle parallel to the vein. The skin of the tail is then pierced and the needle advanced until resistance is no longer felt. The plunger of the syringe should then be aspirated to ensure proper placement of the needle. The use of a needle with a clear or transparent hub will facilitate confirmation of correct placement. Blood backflow into the needle confirms entry into the vein. Alternatively, a butterfly needle with an extension line may be used. The butterfly needle with an extension set precludes the need to hold the tail, needle, and syringe. When using this type of setup, the butterfly needle is attached to an extension set and syringe that is filled with the test compound. The tail may be taped to the table and the butterfly needle is then inserted into the vein, and placement is verified by aspiration on the syringe. Once confirmed, the butterfly needle may also be taped in place. This prevents the needle from pulling out of the vein during dosing. This type of setup can be very useful when administering large volumes of test article as a slow bolus over several minutes or when the test compound may be irritating or mildly caustic. Taping the animal's tail in place prevents the animal from pulling the tail out of the fingers of the technician dosing.

If repeated dosing is to be performed, the initial venipunctures should be performed as close to the tip of the tail as possible. During the injection, if the needle comes out of the vein, a bleb will form under the skin. The needle should be repositioned immediately to prevent infiltration of the solution around the vein. Infiltration of an irritating solution can cause necrosis and make future injections difficult or impossible. Injection of 2 mL/100 g body weight can be accomplished without stress to the rat. Barrow (1968) found that injections of volumes over this amount produced respiratory difficulty and pulmonary edema.

Tail Vein Infusions

Tail vein infusions are convenient because catheter placement can be accomplished without anesthesia. A 23-gauge or smaller needle connected to an extension set is inserted into the tail. The needle and extension set are then secured to the tail with tape. The extension set is attached to a syringe that is placed on a pump and the test compound can be infused. The tail may be taped to a wooden stick or tongue depressor to further protect the needle from being dislodged. The needle catheter is also commercially available and offers the advantage that the needle is removed once the catheter is placed in the vein and may help to prevent further penetration of the vein wall and subsequent perivascular dosing (Rhodes and Patterson, 1979). Advantages that this technique has over permanent indwelling catheters are that the catheter is removed following dosing and will not become occluded and the animal doses do not have to undergo anesthesia and surgical procedure to place the catheter. Permanent catheters have a tendency over time to develop a fibrin flap or become clotted thus losing patency. A major disadvantage is that the animals have to be restrained during the infusion, which may cause stress and alter the results of the study. When using this technique, the duration of the infusion should be limited so that the length of time the animal is restrained is limited.

An alternative technique using the lateral tail vein involves placing a catheter in the vein and wrapping the tail in a similar manner as previously described, and then a lightweight protective cover attached to a tether system is placed around the tail to hold the catheter or needle in place.

Jugular Vein

Although this route has been used for bolus injections, it is most widely used as a site for cannulation for indwelling catheters. The indwelling catheter required surgical implantation under anesthesia.

Bolus Injection Technique

Although injections can be made by exposing the jugular vein by incision, this method is not acceptable for repeated dosing. The jugular vein can be accessed for test compound administration without exposing the vein. The animal can be either anesthetized or restrained on the back. The head is positioned to either the left or the right for access to the respective jugular vein. A 23-gauge needle fitted to a syringe with the bevel up is inserted in a cephalocaudal direction into the angle made by the neck and shoulder. The needle should enter the vein anterior to the point at which it passes between the pectoralis muscle and the clavicle. When about one-half the length of the needle has penetrated the skin, the bevel should be in the lumen of the vessel. Insertion of the needle through the muscle stabilizes the needle and minimizes bleeding. Caution should be used when using this technique as it is considered to be a "blind stick" into the vessel and damage to the vessel may occur. Repeated access of the vessel is not recommended.

Infusion

For the purpose of continuous infusion of the test compound over extended periods of time or for repeated short-term infusions, implanted catheters in the jugular vein may be used. For implantation of a jugular catheter, the animal is first anesthetized and placed in a dorsal recumbency and the surgical site is prepared. A midline incision is then made in the neck and a section of the jugular vein is dissected free. Manipulation of the vein should be limited to prevent vasospasm. A cephalic ligature is then tied and the vein elevated. A small incision is then made in the vein and the catheter is passed into the vein and tied in place. The other end of the catheter is then tunneled

subcutaneously to between the scapulae where the catheter is exteriorized. The catheter should be filled with an anticoagulant solution such as heparin when not in use. When correctly positioned, the tip of the cannula will be at the junction of both venae cavae. If placing catheters into young animals, enough of the catheter should be inserted to allow for the growth of the animal. Care should be taken that the catheter is not inserted too far as the tip may be pushed into the right ventricle of the heart. Improper placement of the catheter may lead to administration of the test compound directly into the heart, which can cause complications.

Similar to the jugular vein, administration of test article via the femoral vein requires an implanted catheter. For implantation of a femoral catheter, the animal is first anesthetized and placed in a dorsal recumbency and the surgical site is prepared. A midline incision is then made in the inguinal area and a section of the femoral vein is dissected free. Manipulation of the vein should be limited to prevent vasospasm. A ligature is then tied and the vein elevated. A small incision is then made in the vein and the catheter is passed into the vein and tied in place. The other end of the catheter is then tunneled subcutaneously to between the scapulae where the catheter is exteriorized. The catheter should be filled with an anticoagulant solution such as heparin when not in use. When correctly positioned, the tip of the cannula will be positioned in the vena cava. For longer-term infusions, the femoral vein catheter may be preferable as patency is easier to maintain and the risk of damage to heart from the catheter is avoided.

Several commercial vendors offer surgical support services and for an additional fee will implant either jugular or femoral catheters. These vendors will typically have a specific methodology for implant, but will accept requests for modifications such as catheter type and exteriorization site. The typical catheter implanted may be manufactured from polyethylene, polypropylene, or silastic. In recent years, manufacturers have developed catheter impregnated or ionically bound with heparin. These catheters may help to prolong the life of the catheter (Joint Working Group, 1993).

The useful lifetime for jugular and femoral catheters is quite variable; the lumen of the cannula may eventually become obstructed by a blood clot or fibrous mass. The position of the tip of the catheter is important. Clot formation is less likely to occur if the tip of the catheter is placed in the venous stream rather than in the jugular vein (Popovic and Popovic, 1960). It is recommended for repeated short-term infusions, that when test article is not being infused, a slow infusion of saline will help to prolong the life of the catheter.

Prior to use, the patency of the catheter should be checked by removing the anticoagulant lock, checking for blood drawback, and then flushing with saline or lactated ringer's solution. Alternatively, patency can be checked by injecting 3–6 mg of pentobarbital solution (0.05–0.10 mL of a 60 mg/mL solution) into the catheter (Weeks, 1972). If the catheter is patent, the rat will lose its righting reflex and become ataxic with 10–15 s of injection. The rat will recover in 10–15 min.

Rats will destroy the catheter if left unprotected or in easy reach of the forepaws. By exteriorizing the catheter between the scapulae, the rat will not be able to chew on the catheter. For the purpose of continuous infusion, several manufacturers have developed tether systems and catheter sheaths made of metal that prevent the animal from chewing on the catheter. These systems typically consist of a jacket with an attached tether that the catheter passes through. The catheter then attaches to a swivel that prevents the catheter from becoming kinked. The swivel then attaches to a second catheter that can be attached to a syringe or pump for administration of the test compound (Davis, 1966; Guo and Zhou, 2003). When performing long-term infusion studies, the effects of the catheter and harness should be considered. Infections, septicemia, a variety of visceral lesions, endothelial lesions, and increased platelet consumption have been observed in cannulated animals (Hysell and Abrams, 1967; Meuleman et al., 1980; Vilageliu et al., 1981). Decreased or erratic weight gains and decreased liver and thymus weights have been observed in tethered animals. These changes may be attributed to the stress involved in chronic tethering of the animals.

An alternative to an exteriorized catheter is to attach a subcutaneous port to the catheter that can be accessed via a transcutaneous needle stick. This type of setup helps to prevent infections

that can occur with transcutaneous catheters. One of the pitfalls of this subcutaneous port is that the port may only be accessed a finite number of time. In addition, care has to be taken to ensure the port and catheter are properly flushed of all test compound and blood as clots cannot easily form. Administration of small volumes of test compound may be accomplished using a subcutaneously implanted osmotic pump. This type of pump is connected to the catheter after being filled with the test compound and implanted in a subcutaneous pocket. This allows for a continuous administration of small amounts of compound without the need for a jacket and tether system.

Saphenous, Lateral Marginal, and Metatarsal Veins

These veins in the leg and foot are easily visualized and can be injected without anesthesia; however, assistance is required. Shaving the area over the saphenous or lateral marginal vein makes visualization easier. During injection, it is necessary for one technician to restrain the animal and occlude the vessel to cause it to dilate. Wiping the skin over the vein with 70% alcohol or with gauze soaked in hot water will help to dilate the vessel and increase the possibility of success. The second technician then performs the injection; a 26–27-gauge needle should be used.

Dorsal Penis Vein

When administering test article via the dorsal penis vein, it is preferable to use anesthesia. Lightly anesthetizing the animals with an inhaled anesthetic such as isoflurane or CO_2/O_2 will prevent the animal from struggling and increase the possibility of a successful injection. This procedure requires two technicians to perform the injection. One technician holds the animal by the skin on the back and the feet and tail. The vertebral column is then hyperextended. The second technician then grasps the tip of the penis between the thumb and forefinger and injects the test solution into the dorsal vein using a 26–30-gauge needle.

Sublingual Vein

While the method of sublingual vein injection has the disadvantage of requiring anesthesia, it only requires one technician. Ideally, the animal should be anesthetized with an inhaled anesthetic such as isoflurane or CO_2/O_2, but injectable anesthesia may also be used. The animal should be placed in a dorsal recumbency with the head toward the operator. The test compound may be administered by holding the tongue between the thumb and forefinger, using a 26–30-gauge needle, the vein is entered at a very shallow angle, and the injection is performed. After completion of the injection, the bleeding can be stopped using direct pressure. Once the bleeding has stopped, a small cotton-wool pledget should be placed over the vein and the tongue placed back in the mouth. The animal will spit the cotton out upon regaining consciousness.

Intraperitoneal Route

Test compounds injected into the peritoneal cavity will be absorbed into the portal circulation and transported to the liver. As a result, the compound will be subjected to the metabolic activity of the liver prior to being circulated to the remainder of the animal. Based upon the level of blood flow and circulatory surface area in the peritoneal cavity, compounds injected intraperitoneally will be absorbed quickly.

Intraperitoneal administration of test compounds in the rat can be performed by one person. The animal should be picked up by the scruff of the neck and back and held firmly in a dorsal recumbency. This position will allow for proper access to the peritoneal cavity. The belly of the animal should be visually divided into quadrants, and a needle (<21-gauge) should be inserted anteriorly
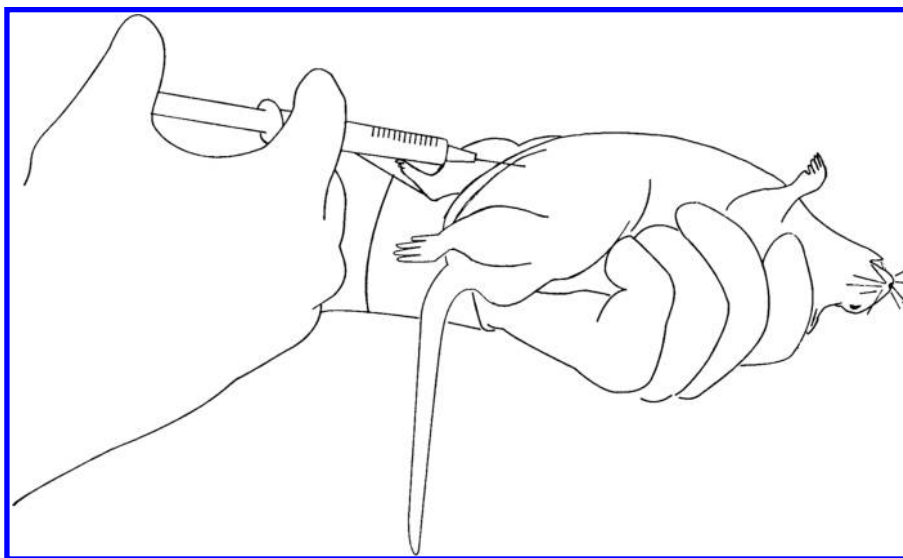
Figure 3.7   Intraperitoneal injection technique.

into one of the lower quadrants just lateral to the midline (Figure 3.7). Aspiration of the syringe prior to injection will help the investigator to determine if the needle is positioned appropriately.

Intramuscular Route

Intramuscular injection of compounds will result in the rapid absorption into general circulation due to the abundant supply of blood vessels. However, the speed of absorption will not be as fast as with an intraperitoneal injection. Acceptable sites in the rat are the quadriceps, the thigh, and the triceps. This procedure can be done with one or two technicians. The selected muscle mass should be stabilized with the thumb and forefinger of one hand while restraining the animal and the needle guided into the muscle with the other hand. A 21-gauge or smaller needle should be used. The needle should be lightly aspirated to ensure the tip of the needle is not in a blood vessel and then the compound is slowly injected. A slow injection with a minimal volume will help to minimize pain. Approximately 1 mL/kg of solution can be injected per site. If larger volumes are required, multiple injection sites should be used.

Subcutaneous Route

Absorption following subcutaneous injection is typically slower than following intramuscular injection. This may be advantageous if a relatively sustained period of absorption is desired. Another advantage of the subcutaneous route versus the intramuscular route is a much larger volume of test compound can be administered. Five to 10 mL can be easily injected with little to no discomfort to the animals. This can be beneficial for test compounds that have limited solubility. Suitable sites for injection are the ventral body, the flank, and shoulders. To perform the injection, the skin is grasped between the thumb and forefinger and raised to make a tent. The needle (<20-gauge) is inserted through the skin to make the injection. Injection sites can be varied for multiple-dose studies where the solution is a potential irritant.

To minimize the stress of manipulation and to provide a means for continuous infusion, a perforated cannula or catheter can be implanted transcutaneously. The cannula can then be secured using

a tether system similar to that used for intravenous infusions. Using this system, the test compound can be infused continuously. Mucha (1980) injected sodium pentobarbital directly into the cannula and showed it was absorbed much more rapidly than following injection. An alternate method of infusion of small volumes over an extended period of time is through the use of an osmotic mini-pump. The pump is filled with the test compound and implanted subcutaneously under anesthesia. These pumps are commercially available and offer the advantage of a continuous infusion at a constant rate without the animals being encumbered with the infusion apparatus. This method would work only when the solutions are stable at body temperature for the duration of the infusion period. In recent years, several types of absorbable microspheres have been developed that can act as carriers for the test compound.

Topical Route

The rat has not traditionally been used as a model in skin irritation or sensitization studies. However, the rat has been used in systemic toxicity studies where the skin is used as a portal of entry for whole-body exposure or in skin painting studies where the carcinogenic potential is being assessed. In a comparison of absolute absorption rates of several compounds, Bartek et al. (1972) found dermal absorption rates in the rat tended to be slightly lower than in the rabbit, and higher than in the monkey, swine, and human.

Exposure in dermal studies is usually to the anterior dorsal portion of the back. The skin should be shaved weekly or 24 hours prior to skin painting. Care should be taken to ensure that the skin is not damaged during shaving, as this can increase the rate of absorption of the test compound. The test area for application should be clearly marked; for repeat dose studies, the area of this site is often 10% of the body area. Usually, 0.25–1.0 mL of the test solution is applied in skin painting studies. The amount of a cream or ointment applied will vary with the test compound and desired total dose administered. Dosing is typically performed every day.

The actual dose in a dermal toxicity study is determined by the amount of compound absorbed; therefore, factors that influence absorption should be considered. Several design features in a topical study may affect absorption; abraded skin will tend to absorb faster than intact skin; test compound may adhere or build up at the site of exposure and may impede absorption (and test compound may be chemically changed owing to exposure to air or light); the test compound may be licked or scratched from the site; and the test compound may be ingested by the animals.

Several techniques have been developed to avoid removal or ingestion of the test compound. For acute studies, Rice and Ketterer (1977) described a cable-type restrainer attached to a stainless steel plate. Loops just behind the front and just in front of the hind legs hold the animal immobile. Other methods may be used to reduce stress to the animals and allow the animal mobility. One method is the use of an "Elizabethan" collar. This is a 4 to 5 cm wide strip of plastic or method that fits around the neck of the animals. This prevents the animal from being able to turn its head to gain access to its back. Consideration should be given to the use of this method as the collar also may prevent the animal from being able to properly eat from a feed jar. A second method is to wrap the animal with gauze and then with plastic wrap. Care should be taken when wrapping the animal to ensure that the animal is not too tight. This type of covered exposure may affect the absorption of the test compound. Other types of harness, collars, and acrylic chambers may be used, and the appropriate technique should be chosen based upon the intended length of exposure and the efficacy with which the technique can be performed by the technicians.

Rectal Route

The rectal route is not a routinely used method of administration in toxicology. However, administration by this route is sometimes required to support drugs given rectally by suppository.

For dosing, the animal is held by the base of the tail and a stainless steel, ball-tipped gavage needle (5 cm) or vinyl tube (6 cm) attached to a syringe is inserted into the rectum. Care must be taken not to damage the rectum when inserting the needle. The syringe should be held lightly: the weight of the needle and syringe propels the needle. The animal can be either awake or anesthetized. If animals are awake, excretion of the unabsorbed test compound may occur. Methods to control excretion have included ligation of the rectum (Nishihata et al., 1984) or various types of septums, which are tied or glued in place (DeBoer et al., 1980; Iwamoto and Watanabe, 1985). Anesthetized animals can be placed on an inclined board to retard expulsion.

An important factor in rectally dosing the rat is that the depth of deposition of the test compound will affect the rat of absorption and should be standardized. Drugs subject to extensive first-pass metabolism, such as propranolol and lidocaine, have been found to be much more bioavailable when injected close to the anus rather than in the upper areas of the rectum (DeBoer et al., 1980; Iwamoto and Watanabe, 1985). The reason for this difference in bioavailability appears to be that the venous return in the upper rectal area is through the upper rectal vein that feeds back into the portal circulation and then into the liver. The venous return in the lower rectum is through the lower hemorrhoidal veins and is not connected to the portal system, but goes directly to the inferior vena cava (Iwamoto and Watanabe, 1985).

Intranasal Route

With the increasing number of drugs being delivered nasally, methods have been developed to support this route. For administration in unanesthetized animals, the appropriate volume of test material is drawn into the tip of a pipette or other appropriate dosing implement. The tip of the dosing implement is placed directly over but not into the nostril to be dosed and the test material is instilled into the nostril. The animal will aspirate the test material into the nasal passage. This can be repeated for the opposite nostril, or the opposite nostril can be used as a control treatment. In the event that the animal sneezes or the test compound is otherwise expelled, the nostril should be retreated.

Inhalation Route

Owing to the complexities and equipment involved in generating, maintaining, and measuring appropriate atmospheres, the inhalation study is one of the most technically difficult to perform. This section is not written to provide a complete discussion of the skills necessary to perform an inhalation study, but will deal with the general considerations about the three major steps in exposing rats by the inhalation route: generation of the test atmosphere, exposure of the test animals, and measurement and characterization of the test atmosphere.

Generation of Test Atmosphere

Test atmospheres that are commonly generated include gases, vapors, aerosols, and/or dusts. Gases are the easiest atmospheres to generate as they can be metered into an airstream, mixed to an appropriate concentration, and then introduced into the test chamber. Vapors are the gas phase of a liquid or solid that has been heated. These can be passed at an appropriate concentration to the exposure chamber in nitrogen or compressed air. Considerations with vapors involve avoiding chemical modification during the heating process and avoiding condensation. Aerosols (stable suspensions of fine solid or liquid particles and dusts and solid particles in air, but not necessarily in a stable suspension) are more difficult to generate. A variety of generators have been developed to nebulize or disperse the particles into the air. Once generated, aerosols and dusts are mixed to appropriate concentrations and introduced into the exposure chamber. Considerations involved

in aerosol and dust generation include particle size, size distribution, and particle shape, as these parameters will primarily determine where in the respiratory tract the particles will be deposited and the rate at which they will be absorbed. As discussed earlier, rats are obligate nasal breathers. As a result of this characteristic and their smaller nasal passages, particulates above 1 µm are essentially excluded from rodent lungs.

Exposure of Test Animals

Exposure of animals can be performed using several different chamber designs. The most common three are whole body, nose only, and head only. Design features common to these chambers include the following: they are made of nonreactive material and have provisions for easy access to and viewing of the animals; they are designed to ensure a uniform flow of test material to the animals; and they have a number of ports for monitoring test compound concentrations, chamber temperature, humidity, and pressure. The advantages of head- and nose-only chambers are that they require much less compound, compound exposure is limited to the nose or to the head, and the animals are more accessible for physiological measurements. A major disadvantage is that the animals must be restrained in tubes and, therefore, exposure times must be limited to minimize stress and possible overheating. Advantages of the whole-body chamber are that loading and unloading are much less labor intensive than other designs and the animals are maintained in cages, allowing longer exposure times. Disadvantages of whole-body chambers are that aerosols and dusts deposit on the fur and may be eaten or absorbed dermally and the size of chamber requires a much larger amount of test compound.

In the exposure process, animals are placed into chambers and exposed for a predetermined amount of time to the test atmosphere. Generally, exposures in head- and nose-only chambers are limited to a set number of hours per day, whereas whole-body exposures can be continuous, even chronic. Dosages in inhalation studies are generally stated on the basis of the period of time at a stated atmosphere concentration. Estimation of actual dosage received is complex and is based on the physiology and anatomy of the rat and upon several characteristics of the atmosphere.

Measurement and Characterization of the Test Atmosphere

An important part of the process is documenting the atmosphere to which the animals were exposed. Measurements are made to determine characteristics, including the concentration of the atmosphere, homogeneity of distribution, and, in aerosol and dust studies, the size of the particles generated. Samples should be drawn from a number of sites within the chamber to determine homogeneity. Gases and vapors can be analyzed using chromatography or spectrophotometry. Aerosols and dusts are collected and then measured using methods such as sedimentation, filtration, centrifugation, impaction, thermal or electrostatic precipitation, or by optics.

Intratracheal Administration

An alternative to inhalation administration is to instill the test compound into the trachea of the animal. Techniques have been developed to instill the test compound into the trachea safely and repeatedly. To perform the procedure, the animal must first be anesthetized, preferably with a gas anesthesia such as isoflurane. Once anesthetized, a speculum is inserted into the mouth and passed into the trachea. A syringe and needle with a 5 cm piece of tubing is used to instill the test compound. The tubing is then passed over the speculum and the test compound is administered. Volumes should be limited to 2 mL or less of test compound.

Data Collection Techniques

Observations and Physical Examinations

Rats are routinely monitored during toxicology studies to assess their general health and to define the effects of the test article. In acute and subchronic studies, animals may be observed frequently in an effort to define short-term pharmacologic changes induced by the test compound, which may become apparent at peak blood levels. Specifically in acute toxicology studies, clinical observations will help to establish an MTD. In chronic studies, these observations are critical in tracking tumor development and for determining animals in extremis, which should be euthanized for humane reasons and to prevent autolysis and tissue loss. Arnold et al. (1977) contains a useful description of a clinical assessment program for chronic studies.

Daily observations are performed first thing in the morning and last thing before leaving in the afternoon to assess the health of the animals and identify animals that may be in extremis. In this observation, behavioral status, respiratory signs, skin, eyes, and excretory products are noted. Care should be taken to disturb the animal as little as possible, as this may induce stress and affect the animal's behavior. The animal should be picked up and examined more closely if abnormalities are detected. Special attention should be paid to the amount of feces present, since a decrease in fecal output may be the first signs of a watering system malfunction. In acute studies or where pharmacological effects are expected, animals may be examined continuously or at peak plasma levels.

A more thorough physical examination should be done weekly. Each animal is taken from its cage and placed on an examination table where respiration, behavior, general appearance, and locomotion are observed. The technician should then pick up the animal; examine its body orifices, skin, and coat; and perform a palpation of the trunk and limbs to check for tumors. The detection and tracking of the size and fate of masses (potential tumors) are essential for carcinogenicity studies.

Animals that have experienced severe weight loss over the previous week, a progressive decline in weight over several weeks, or other severe clinical signs should be observed more frequently and marked for possible euthanasia. Body and feeder weights can be measured as part of the physical examination or as a separate function. Performance of these operations as a part of physical examinations will minimize animal handling and potential stress. If the operations are combined, it is important that the physical examinations be done completely and not rushed.

Neurobehavioral Examination

Neurobehavioral examinations are included in toxicology studies to assess the behavioral and neurological effects of test compounds. These examinations, which may be done as part of acute or repeat dose toxicity studies or study specifically designed to assess just the neurobehavioral effects, typically involve screens consisting of an abbreviated FOB and some measure of locomotor activity. The EPA has written guidelines on the design and conduct of these studies (OECD, 1995, 1997). To meet EPA guidelines, the screen is performed prior to the start of treatment and then periodically during the course of treatment. It may be performed as a separate study, on satellite groups of animals in conjunction with the main study or on animals in the main portion of the toxicology study. Recently, the ICH has adapted guideline requiring neurobehavioral assessment of all new pharmaceutical prior to initiation of Phase I clinical trials. To meet ICH guideline, a more formal FOB should be conducted and may be conducted on main study animals or may be conducted as a separate study (ICH, 2000). Where initial screens indicate the possibility of a test compound–related change, a more specialized series of test may be performed to assess the nature of the effect and the extent of the central nervous system involvement. These secondary

tests evaluate motor and sensory functions as well as cognitive ability. Examples of secondary test include sensory-evoked potential experiments and schedule-controlled behavior studies. Descriptions of these secondary tests can be found in Annau (1986).

Functional Observational Battery

The typical functional observation battery (FOB) includes observation of home cage and open-field activities as well as measurements of reflexive, physiological, and neuromuscular function (Moser et al., 1988, 1996) as outlined in Table 3.15. Observations and measurements that have become standard for an FOB evaluation are in Table 3.16. The order of measurement should be consistent, progressing from the least interactive to the most interactive measurements. Home cage observations are made first. Assessments of posture, clonic movements, tonic movements, and palpebral closure (if the animal's eyes can be seen) are taken prior to removing the animal from its cage. However, it may be necessary to pull the cage from the cage bank or remove the cage cover in order to see the animal's eyes. The animals are then transferred to the open field. During the transfer, certain physical observations are made. The technician removes the animal from the cage. The technician holds the animal and notes increased or decreased body tone as well as such observations as bite marks, soiled fur appearance, missing toe nails, emaciation (shallow stomach, prominent spinal vertebrae), or death. In addition, observations of lacrimation, palpebral closure, piloerection, exophthalmos, and salivation are also made. The animal is then placed in the open field apparatus for a set period of time. Measurements of rearing, urination, and defecation are made immediately at the end of the assessment period. Assessment of clonic movements, tonic movements, gait, mobility score, arousal, respiration, stereotypic behavior, and bizarre behavior may be made immediately after the time period has ended, or the technician may continue to observe the animal for a longer

Table 3.15 Functional Observational Battery in Rats

	Number of Animals	
	Males	**Females**
Control	10	10
Low dose	10	10
Mid dose	10	10
High dose	10	10

Dosing: The test article will be administered by the required route.

FOB evaluations will be conducted prior to dosing, at the estimated time of peak effect, and 24 hours post-dose. The evaluations are as follows:

Home cage observations: Assessments of posture, clonic movements, tonic movements, and Palpebral closure (if the animal's eyes can be seen) are taken prior to removing the animal from its cage.

Handling observations: Observations of ease of removal, handling reactivity, lacrimation, palpebral closure, piloerection, and salivation are made upon removal of the animal from the home cage.

Open-field observations: The animal is placed in the center of an open-field testing box (measuring 20″ × 20″ × 8″). Clean absorbent paper may be used to cover the bottom of the box if required by protocol. Using a stopwatch, the animal's stay in the box is timed for 3 min. Measurements of rearing, urination, and defecation are made immediately at the end of the 3 min. Assessment of clonic movements, tonic movements, gait, mobility score, arousal, vocalization, respiration, stereotypic behavior, and bizarre behavior may be made immediately after the 3 min have ended, or the technician may continue to observe the animal for a longer period of time to allow for more accurate assessment.

Sensorimotor observations: The approach response, touch response, click response, and tail pinch response (stimulus reactivity tests) are performed while the animal is in the open-field apparatus, after the 3 min time period is over and all other measurements have been recorded. The animal is removed from the open-field apparatus for the pupil response, righting reflex, thermal response, hind limb splay, and grip strength measurements.

Physiological evaluations: The animal's body weight and rectal temperature are measured and recorded.

Clinical examination: Following each FOB assessment (additional observations will be conducted prior to dosing for locomotor activity animals, if requested).

Table 3.16   FOB

Home Cage	Open Field	Manipulative	Physiological and Neuromuscular
Posture	Rearing	Ease of removal from cage	Body weight
Clonic movements	Urination	Handling reactivity	Body temperature
Tonic movements	Defecation	Lacrimation	Hind limb extensor strength
Palpebral closure	Clonic movements	Palpebral closure	Grip strength
	Tonic movements	Piloerection	Hind limb splay
	Gait	Exophthalmos	
	Mobility	Salivation	
	Ataxia	Approach response	
	Arousal	Touch response	
	Vocalizations	Click response	
	Respiration	Tail pinch response	
	Stereotypy	Pupil response	
	Bizarre behavior	Eye blink response	
		Forelimb extension	
		Hind limb extension	
		Righting reflex	
		Thermal response	

Sources: Haggerty, G. C., *J. Am. Coll. Toxicol.*, 8, 53, 1989; Moser, V. C., *J. Am. Coll. Toxicol.,* 8, 85–93.

period of time to allow for more accurate assessment. When the open field assessment has been completed, the animal is removed from the open field apparatus for the approach response, touch response, click response, tail pinch response, pupil response, righting reflex, thermal response, hind limb splay, and grip strength measurements. After completion of the manipulative assessments, the physiological evaluations are completed.

Procedures used during the performance of an FOB must be standardized because some observations made have a subjective component. If at all possible, a single observer should be used throughout a single study. If not possible, a single observer should conduct all assessments of an animal. In addition, technicians should be blinded to the treatment conditions for each animal.

Locomotor Activity

Methods used for recording motor activity include direct observation and automated techniques such as photocell devices and mechanical measurements (MacPhail et al., 1989). In direct observations, the observer can make quantitative measurements of the frequency, duration, or sequencing of various motor components of behavior or qualitative records on the presence or absence of certain components of activity. Photocell devices record the number of times an animal interrupts a beam in specially designed chambers. In mechanical chambers, the animal's movements result in a vertical or horizontal displacement of the chamber; records are kept of the chamber's movements. There are advantages and disadvantages of each technique. In direct observation, record can be made of behavior, such as convulsions, that may not be observed when using the photocell or mechanical method. A disadvantage of the direct observation method is that the animal may be influenced by the presence of the observer. Advantages to the photocell and mechanical methods are that the data are captured electronically, the observer does not have to be present, and the computer system can graphically present the data in the form of lines crossed or a map of the activity.

To make activity determinations, an animal or a group of animals are put into an observation or recording chamber, and activity is recorded for a specific period of time. Since activity will normally decline over the course of the session, the length of the observation period is important.

The EPA guideline (EPA, 1995) specifies that activity should approach asymptotic levels by the last 20% of the session. Haggerty (1989) used a 15 min recording session, accumulating data over three 5 min intervals. Since a large number of environmental conditions can affect motor activity, e.g., sound level, cage design, lighting, temperature, and humidity, or odors, it is important to minimize variations in the test environment.

Cardiovascular Parameters

Examinations of the cardiovascular system may be scheduled into toxicology studies or performed when the cardiovascular system is a suspected target of the test compound. The ICH has adapted guideline requiring cardiovascular safety assessment of all new pharmaceuticals prior to initiation of Phase I clinical trials. This guideline recommends that this assessment be conducted in a nonrodent species, but cardiovascular assessment can be performed for screening purposes or as additional support data.

Electrocardiography

Although the dog has traditionally been the species of choice in toxicology studies of effects of ECG, research with the rat has progressed and increased over the years. Detweiler et al. (1981) provides an excellent review of the use of electrocardiography in toxicology studies in the rat. This section will present a general discussion on the aspects of the recording methods and interpretation of the ECG in the rat.

Recording Methods

Restraint

One of the disadvantages of traditional methods of studying ECGs in rats is that it is difficult to keep the animals still while recording. It is important that the animal remain in a constant position during the procedure using skin leads to avoid muscular artifact in the ECG. Various forms of restraint have been tested, each of which requires acclimation to the procedure prior to evaluation. These methods included restraining the rat in a supine position using rubber gloves (Hundley et al., 1945) and pinning the animal to a board, boards with clamps, and plastic tubes with slits on either side that allow for placement of the electrodes (Zbinden et al., 1980). Various forms of anesthesia have also been evaluated.

No conclusion has been reached about the best method of restraint. The basic concerns are that manual methods and physical restraint require acclimation to allow the animal to become accustomed to the procedure, and tracings can be reasonably free of muscular artifact. Also, varying pressures of clamps or handling during the restraint may affect the results. The use of anesthesia has been shown to produce changes in the ECG, and the possibility of drug interactions between the anesthetic and the test compound may occur.

Position: The most common positions are the prone or ventral recumbency position when animals are awake and the supine or dorsal recumbency position when anesthetized. Beinfield and Lehr (1956) compared the positions and concluded that the prone position produced and increased R wave, and it avoided unfavorable cardiac rotation and an undesirable variation in the projection of the special QRS loop.

Tethered: Robineau (1988) developed an electrode system that can be implanted subcutaneously a few days prior to recording. The device has a disconnect that is exteriorized between the scapula. The advantage of this system is that a cable can be connected to the plug and the ECG can be taken

in unrestrained rats. The disadvantage of this method is that this method should only be used for short-term studies as the implant provides a source for infection in the animal.

Telemetry: Recently, techniques have been developed for monitoring cardiovascular parameters via telemetry in the rat (Ichimaru and Kuwaki, 1998; Kramer et al., 1995; Kuwahara et al., 1994). Totally implantable battery-operated systems have been developed that can monitor several physiological parameters including ECG. The implants are available with ECG leads that can be positioned in the lead II configuration for monitoring ECG continuously for extended periods of time in a freely moving animal. As the leads are attached subcutaneously to the musculature, the signal is of a higher quality than skin leads. Also, the animal is not affected by the observer, as the animal can be monitored remotely. One of the disadvantages is that these implants are only designed to monitor a single lead.

Leads: Most investigators use Einthoven's bipolar limb lead system, lead I (right and left foreleg), lead II (right foreleg and left hind leg), and lead III (left foreleg and left hind leg), with and without the augmented unipolar limb leads aVR (right foreleg), aVL (left foreleg), and aVF (one of the hind legs). Since foreleg position can alter the scalar ECG wave amplitudes, investigators must standardize foreleg positions during recording. When implanting telemetry leads, the leads are placed on the right clavicle and the most caudal rib on the left side in a modified lead II configuration.

In the past, various types of leads have been used to connect the ECG wires. These included the use of hypodermic needles inserted under the skin, small gauge insulated copper wires wrapped around the shaved distal portion of the limbs, or alligator clips. In addition, platinum-tipped pin electrodes are commercially available that provide a good-quality signal with limited discomfort to the animal.

ECG Waveform

A diagrammatic example of a normal rat ECG is illustrated in Figure 3.8. The major points to notice about the rat ECG are that the conventional waves of the mammalian ECG (P, QRS, and T) are all identifiable in the rat ECG, there is no isoelectric line during the electrocardiographic complex, and there is no ST segment. The duration of the standard intervals evaluated in the ECG of the rat is as follows: P, 10–20; PR interval, 35–50; QRS complex, 12–25; and QT interval, 38–80 ms (Detweiler, 1981). The duration of the intervals is related to the heart rate; as the heart rate increases, the intervals shorten, and as the heart rate slows, the intervals prolong. These intervals can also be affected by administration of test compounds and are the basis for the requirement to assess the effects of a test compound on cardiac function. Specifically, prolongation of the QT interval has been correlated with a phenomenon called torsade de pointes or sudden cardiac death. Several classes of compounds, such as antihistamine and Ca^{2+} channel blockers, have been shown to prolong the QT interval (Gras and Llenas, 1999). Each wave of the ECG represents either a depolarization or repolarization of the atria and ventricles of the heart. For example, the P wave is an electrical representation of the depolarization of the right atria, and the T wave is an electrical representation of the repolarization of the left ventricle. Spear (1982) provides a more in-depth discussion of the waveforms and the electrophysiology of the heart. Several computerized systems have been developed that are capable of recognizing the independent waveforms and measuring the intervals. Caution should be used when using these systems as they are typically programmed to recognize a normal ECG, and the presence of arrhythmias may be missed or interval may not be measured correctly.

Heart Rate

The heart rate can be calculated from standard limb lead ECGs by measuring the distance between the two peaks of the R wave. This distance is then divided by the chart speed (i.e., 50 mm/s)
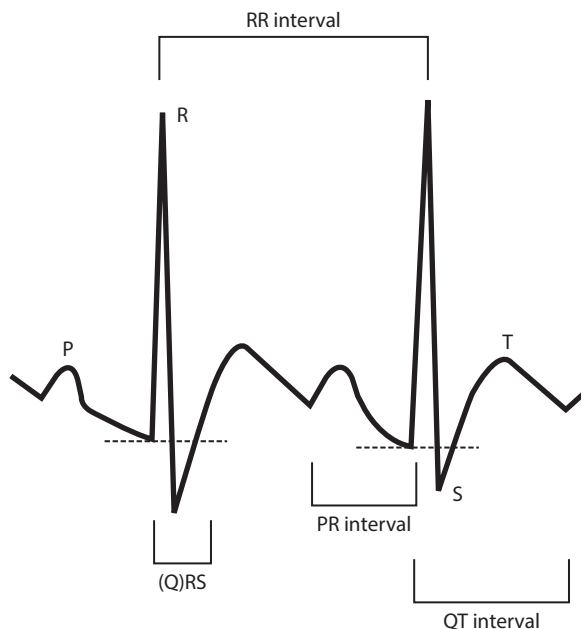
Figure 3.8   Diagrammatic representation of rat ECG waveform with intervals indicated.

to calculate the RR interval in seconds. This is then divided into 60 s to calculate the heart rate in beats per minute. Using the following formulas, the heart rate of an RR interval of 10 mm measured on a chart printed at 50 mm/s = 300 beats/min.

$$\text{RR interval (mm/beat)}/\text{Chart speed (mm/s)} = \text{RR interval (s/beat)}$$

$$(60\ \text{s/min})/\text{RR interval (s/beat)} = \text{Heart rate (beats/min)}$$

Many environmental things can affect the heart rate of the rat, such as excessive manipulation, technicians the animal is not familiar with, new environments, etc. Therefore, when evaluating ECG using a restrained method, it is important to acclimate the animal to the test procedures prior to starting. Detweiler (1981), in a review of the literature, found that published heart rates for rats varied between 250 and 750 beats/min. Awake, restrained adult rats had heart rates from 330 to 600 beats/min, and well-acclimated restrained adult animals had heart rates from 250 to 350 beats/min. Heart rate in unrestrained telemetrized animals has been reported to be between 225 and 350 beats/min (Guiol et al., 1992).

Blood Pressure

During the conduct of a toxicology study, it may be necessary to monitor blood pressure. This can be done using either indirect or direct methods. Caution should be used when using indirect methods as the values obtained may be variable. The direct method involves the implantation of an arterial cannula for the measurement of blood pressure. This method is more reliable, but has a limited period of time during which they may remain patent. It is recommended that for definitive assessment of the hemodynamic effects of a test compound, a nonrodent species such as a dog or a nonhuman primate be used.

Indirect Measurement

Indirect methods of blood pressure measurement detect systolic blood pressures by the occlusion of arterial inflow of blood and the subsequent detection of the pressure at which the first arterial pulsation occurs. The two places where indirect measurements can be made on the rat are the tail and the hindpaw.

Tail cuff method: The tail cuff method monitors pressures in the ventral caudal artery. In this method, the animal is put into a restrainer that allows for free access to the tail. An inflatable cuff is then placed around the base of the tail, and the pressure is increased until flow stops; the pressure is then slowly released until flow resumes. The cuff pressure at the time when flow resumes is the systolic blood pressure. Various methods have been used to determine when this occurs.

Because the caudal pulse is rather weak, preheating of the animals in boxes at temperatures of 30°C–42°C for periods up to 10 min may be necessary to dilate the caudal artery. This technique should be used with caution, as previously discussed changes in body temperature can have widespread effects on the animal and may produce unexpected test compound effects.

The placement and width of the tail cuff are important. There is a gradient in pressure along the caudal artery that amounts to 4.5 mmHg/cm. For this reason, the cuff should be placed close to the base of the tail and this should be standardized. If multiple reading will be done over time, marking the placement of the cuff with an indelible marker will help to standardize placement. In addition, variation in the width of the rubber tubing can be a source of error. Bunag (1973) found that the most accurate readings were given by a 15 mm cuff; shorter cuffs gave falsely elevated readings and longer cuffs gave low readings.

Hindpaw method: Measurement of blood pressure in the hindpaw does not measure the pressure in a specific vessel. In this method, the animal is placed in a restrainer, as in the tail cuff method. A pressure cuff is placed around the ankle to occlude blood flow, and blood pressure is measured as the cuff pressure is released and blood flow returns. As in the tail cuff method, several techniques have been used to determine the return of blood flow. These include visual observation (Griffith, 1934), photoelectric cell (Kersten et al., 1947), and oximeter (Korol and McShane, 1963). The advantage of the photoelectric and oximeter methods is that they do not require preheating to dilate the vessels of the hindpaw. The oximeter method measures mean arterial pressure rather than systolic pressure. None of the indirect measurements are able to evaluate the complete hemodynamic cycle. They evaluate only systolic or mean arterial pressure, but not systolic, diastolic, and mean arterial pressures together.

Direct measurement: The direct measurement techniques involve the cannulation of an artery with the blood pressure being determined with a manometer or transducer connected to the free end of the cannula. This is true for types of direct measurement including telemetry. Surgery and cannula placement utilize similar techniques as those previously described for placement of a venous catheter. In short, the artery is isolated and a small incision is made in the femoral artery. The catheter is then inserted into the hole and passed into the abdominal aorta. The carotid artery may also be used for this procedure, but care should be taken not to be inserted too far as the catheter may be passed into the left ventricle of the heart. If this occurs, the blood pressure waveform will change in appearance. The left ventricular waveform has a similar systolic pressure as an arterial blood pressure waveform. But the diastolic pressure is much different. If the catheter has been placed in the left ventricle, the diastolic pressure will be 0 mmHg or slightly negative. If this occurs, the catheter should be backed out into the aortic arch.

Where chronic use is desired, cannulas are typically run subcutaneously and exteriorized between the scapula or at the back of the head. A carotid artery catheter can be expected to remain patent for 3–5 weeks (Andrews et al., 1978; Ross, 1977), while an abdominal aorta catheter may remain patent for several months. Care should be taken when using arterial catheters for long

periods of time as fibrin deposits can build up on the catheter or clots may form in the catheters. The risk exists that these deposits or clots could be expelled during the flushing of the catheter or during normal movement of the animal. These clots or deposit once free may occlude other vessels downstream and in the case of a carotid catheter may cause a stroke to occur.

Blood Collection Techniques

Blood samples are routinely collected in safety studies to determine (1) direct test compound effects on the blood or bone marrow; (2) effects on other organs as indicated by the contents of the blood, for instance, leakage enzymes such as aspartate aminotransferase; and (3) blood levels of the test compound or its metabolites. A variety of techniques have been described for the collection of blood from the rat. The choice of a specific technique may depend upon factors such as (1) the volume to be collected, (2) if the animal is to survive the procedure, (3) the frequency with which samples will need to be collected, (4) whether anesthetics can be used, (5) likelihood of the animal surviving the procedure, and (6) the impact of organ damage resulting from the procedure. An adult rat has a blood volume of about 50 mL/kg; approximately 10% of the total blood volume can be collected from the rat in a single draw without adversely affecting hematology parameters. For longer-term studies, 10 mL/kg per 2 weeks is a reliable guideline for volume of blood drawn. If these volumes are exceeded, hematocrit and red cell mass may be reduced upon evaluation (Diehl et al., 2001).

Technique, anesthetic used for blood draws, and treatment of the animals (i.e., fasted or not fasted) should be standardized throughout a study if repeated samples are being taken. The technique, anesthetic, and handling of the animal may produce the effects of the hematology or clinical chemistry parameters to be evaluated.

Retroorbital Plexus

The retroorbital plexus is a commonly used site for periodic sampling during the course of a study. This method has been shown to be a reliable method for the repeated collection of blood samples. Collection from this site should always be conducted under anesthesia to reduce pain and stress to the animal. Light anesthesia with a mixture of carbon dioxide and oxygen will minimize struggling of the animal and will help to ensure a quick collection with little injury to the animal.

Blood is collected using a microcapillary tube or the fine end of a Pasteur pipette. The tube is inserted into the orbit of the eye at an anterior angle formed by the lids and the nictitating membrane. A short thrust past the eyeball will make the tube enter the slightly resistant horny membrane of the sinus. The tube may be rotated slightly as it is inserted. Once the sinus has been punctured, blood will fill the tube. Once the tube is filled, the blood may be allowed to drip out of the end of the tube into an appropriate collection tube. If the flow stops, the tube may be pulled out or advanced slightly to reestablish flow.

In the hands of an experienced technician, there is minimal risk to the animal. Studies have shown that repeated collection of blood from the retroorbital sinus can produce histological and behavioral changes that may require an animal to be removed from the study (McGee and Maronpot, 1979; van Herck et al., 1998). Several serious side effects of this collection method have been documented. These include retroorbital hemorrhage, corneal ulceration, keratitis, pannus formation, damage to the optic nerve, and fracture of the orbital bones. When designing the study, the method of blood collection should be taken into consideration. If an important endpoint of the study is ophthalmological examinations, this method should not be used. In addition, anesthetization of the animals may affect other study endpoints.

Tail

A tail vein bleed offers a visible target and is of minimal risk to the animal. Blood will flow faster if the tail has been warmed causing vasodilation. This may be accomplished by dipping the tail in warm water (40°C–45°C), placing the animal in a warming cabinet for 5–10 min, or warming the tail with a heat lamp. This method does not require that the animal be anesthetized, but the animal should be restrained such that the tail is held immobile. Several methods may be employed to collect blood from the tail. These include clipping the end of the tail off, vein puncture, or artery puncture.

Tail Clip

For this method, the animal may be lightly anesthetized or placed in a restraint tube. To collect blood, 2–3 mm of the distal part of the tail is amputated with sharp scissors or a scalpel blade. Three to 4 mL of blood can be collected in 20–30 s from a 200–250 g rat. When collection has been completed, the cut surface may be cauterized with a hot spatula or glass rod. This method produces a reliable volume of blood, but should not be used for repeated sampling over extended periods of time.

Venipuncture

Animals should be restrained in a holder that allows complete access to the tail. The tail should then be cleaned, and pressure may be applied to the base of the tail causing the vein to dilate. The vein should be punctured using a 21-gauge needle, and either the blood can be slowly withdrawn into a syringe through the needle or the needle may be removed and the tail allowed to bleed freely. The use of a butterfly needle may help facilitate the collection of blood as it is less likely to be dislodged if the tail moves. Collection of blood from the tail vein typically yields samples of between 0.5 and 1.0 mL. Upon completion of sample collection, the needle should be withdrawn and pressure applied to the tail to stop the bleeding (Frank et al., 1991).

Arterial Puncture

This method is conducted in a similar manner to a venipuncture in that the animal is placed in an appropriate restraint tube and the tail is then cleaned. A 21-gauge needle is then inserted into the artery in the midventral surface of the tail close to the distal end. The blood may be then withdrawn into a syringe or allowed to flow freely into a collection tube. The animals' blood pressure will ensure the blood continues to flow until pressure is applied to the wound.

Cardiac Puncture

This method should always be performed under general anesthesia and should not be used as a survival collection technique. This technique offers a rapid method for collection of a large volume of blood from the rat. It is possible to collect between 5 and 7 mL from a 300–350 gram rat using this technique, and it is possible to exsanguinate the animal using this method.

To collect blood using this method, the animal should be anesthetized with a combination of carbon dioxide and oxygen or carbon dioxide alone. The animal is then placed in a dorsal recumbency and the heart is located. The heart may be located by placing the index finger over the fourth and fifth left ribs and the thumb on the right side of the thorax. The collection needle (25–26-gauge, 1–2 cm long) should be inserted at a 45° angle into the heart. Once the needle is introduced, the syringe should be aspirated slightly to produce a vacuum. The needle is then advanced until blood is obtained.

Abdominal Aorta and Vena Cava

Collection from the abdominal aorta offers a convenient way to exsanguinate an animal and obtain a maximal amount of blood. To perform the procedure, the animal is anesthetized and the aorta exposed by dissection. A section of the aorta distal to the diaphragm is then exposed and the proximal end is clamped. The aorta is then cut and the distal end placed in a collection tube and the clamp is released. This method will allow for collection of a maximal amount of blood in a short period of time. This method should only be used as a terminal procedure. Alternatively, the aorta may be accessed using a needle or a butterfly needle, and samples may then be collected into a syringe.

Winsett et al. (1985) described a method of repeated sampling from the vena cava of conscious rats. An assistant holds the animal while the operator grasps the animal just below the last rib. The needle is inserted 1 cm to the right of the spinous process of the first lumbar vertebra at a 45° angle until the needle touches the bone. The needle is then slightly withdrawn and then advanced to a slightly shallower angle to miss the bone and access the vena cava. The maneuver of first identifying the bone is essential to the procedure. This procedure may be used for repeated collection, but care should be taken to ensure the animal does not struggle during the collection. If the animal struggles, the needle may lacerate the vena cava causing the death of the animal.

Jugular Vein

The jugular vein provides a means for chronic blood sampling that is of low risk to the animals' health. This method does not require the use of anesthesia and can be accomplished through the proper restraint and positioning of the animal.

The unanesthetized method requires two technicians to perform the collection. The animal is placed on a restraint board in a dorsal recumbency. The forelimbs are tied down to the board, and one technician holds the hind limbs of the animal. The second technician grasps the animals head and turns it down and away from the desired collection site, right or left jugular. The needle (21-gauge) is then inserted into the middle of the triangle formed by the neck, shoulder, and clavicle, parallel to the body. As the needle is inserted, the syringe is aspirated until blood is observed in the syringe. The collection is then completed and the site is held off for approximately 30 s. It is important that the technician holding the hind limbs of the animal continually observes the respiratory rate of the animal. If the head is turned too far or is held in the wrong position for too long, the animal may go into respiratory distress. This method may be used for repeated collections with high level of success.

Alternatively, the animal may be anesthetized and the jugular vein may be surgically exposed. The animal should be prepared using standard aseptic technique and the ventral neck should be shaved. The jugular vein may be exposed by incision of the skin and dissection of the subcutaneous tissues. A needle (20-gauge) may then be inserted into the vein through the pectoral muscle and directing the needle toward the head. Inserting the needle through the muscle will help to stabilize the needle during the collection. Once the needle is in place, the blood should flow freely with little to no aspiration. Care should be taken when aspirating the syringe as too much pressure will cause the vein to collapse. Once the collection is complete, the incision can be closed with a wound clip. This method may be used as an alternative if the animal does not require serial bleeds or as a method to replace a terminal bleed. If the procedure is used for a terminal bleed, strict aseptic technique is not necessary.

Proximal Saphenous and Metatarsal Vein

A small amount of blood (0.1–0.2 mL) can be collected from animals at minimal risk to their health utilizing the proximal saphenous and metatarsal veins. No anesthesia is required.

Proximal Saphenous Vein

The inner aspect of the thigh of the hind limb should be shaved free of hair. While one technician holds the animal and compresses the inguinal area to dilate the vein, a second technician creates a longitudinal nick in the vein with a 20-gauge needle or a hematocrit lancet. The blood can then be collected into heparinized capillary tubes. This method works well for repeated sampling of small amounts of blood, but would not be appropriate for blood volumes required for evaluation of clinical pathology parameters.

Metatarsal Vein

This procedure can be conducted with or without an assistant. The animal is retrained and a nick is made in the vessel with a needle. The blood can then be collected into a capillary tube or through the needle into a syringe.

Sublingual Vein

When using the sublingual vein for blood sampling, the animal should be anesthetized and then cradled in the palm of the hand. By holding the animal's head between the thumb and index fingers, the head can be stretched back and the skin of the face pulled backward. This will force the mouth open and the tongue against the palate. The right or left vein should be cut with iris scissors and the animal held such that blood drips into the collection tube. The bleeding can then be stopped by applying pressure with a gauze pad or cotton-tipped applicator.

Decapitation

Decapitation should only be performed by trained technicians with the appropriate equipment. There are several commercially available small animal guillotines that should be used to perform this technique. This technique is appropriate when a maximal blood volume is desired and contamination of the sample is not considered to be an issue. To perform this method, the head is first removed and the animal is held over the collection vessel and arterial and venous blood is allowed to drain from the body.

Cannulation

While the blood collection methods described earlier will provide sufficient volumes and quality of sample for the majority of toxicology studies, specific protocols may require blood to be samples from animals that have been subjected to a minimum handling or from specific sites within the body of the animal. Cannulation of a specific artery or vein will typically meet this requirement, though in rodents, the usable life span of the cannula is limited by the length of time the cannula remains patent. Yoburn et al. (1984) compared jugular, carotid, and femoral cannulas for long-term sampling of blood. They found the femoral artery cannula was preferable in terms of patency and postsurgical weight loss. Collection from a cannula is the same regardless of implant site. The cannula is typically exteriorized between the animal's scapula and a stylet is inserted into the end of the cannula. For the purpose of collection, the stylet is removed and a needle is inserted into the cannula with a syringe attached. The heparin lock is then drawn out of the cannula until blood is observed in the syringe. The syringe is then removed from the needle and a new syringe is used to collect the sample. The collection syringe is then removed from the needle and replaced with a syringe filled with the desired solution for locking the cannula, typically a heparin dextrose solution. Once the cannula is flushed and locked with the heparin

solution, the stylet is replaced. Using the appropriate technique for flushing and locking the cannula is key to maintaining the patency of the cannula.

Jugular vein. The cannulation procedure is the same as previously described for infusion techniques. The cannula has been found to remain patent for variable period of time, and the length of time the cannula remains patent is directly related to the skill of the technician collecting the samples. It is important to remember that a cannula placed in the venous system can easily develop clots in the cannula if not flushed and locked properly. Various methods of anchoring the cannula to the rats' back or head have been developed for ease of sampling or as a connection point for continuous infusion. Each of these methods has been developed such that the exteriorized cannula is positioned such that the animal cannot gain access to it.

Inferior vena cava. The inferior vena cava appears to provide a site for long-lived cannulas. The cannula can be either surgically placed directly into the vena cava through an abdominal surgery or advanced into the vena cava from the femoral vein. Either way, the cannula has been shown to remain patent for months (Kaufman, 1980). In either implantation, the cannula is then tunneled subcutaneously and exteriorized between the scapulae in the same manner as the jugular vein cannula.

Abdominal aorta. The most common method for placing a cannula in the abdominal aorta is via the femoral artery. The cannula is inserted into the femoral artery and advanced to the level just above the kidneys. The opposite end of the cannula is then tunneled to and exteriorized between the scapulae. This is a minor surgical procedure for the animal, and the animal easily recovers. Similar methods are employed when collecting blood from the arterial catheter as those used for the venous catheter. Caution should be taken when removing the stylet from the cannula, as a cannula in the arterial system is under pressure. If not properly prepared, the animal could quickly lose a large amount of blood. It is recommended that the technician place a clamp on the cannula prior to removal of the stylet. Once the blood collection is complete, the cannula should be thoroughly flushed with saline prior to locking the cannula. The tip of an arterial cannula is positioned such that it is against the flow of blood. Due to this, clots and fibrin deposits develop easily on the end of the cannula limiting the usable life.

Subcutaneous ports. An exteriorized cannula is a source of contamination and infection and is subject to destruction by the animal or other animals if group housed. Several types of subcutaneous ports have been developed for implantation along with the cannula. These ports are designed such that test materials may be injected or infused through them or blood may be collected from the port. In most cases, the port is implanted subcutaneously on the dorsal side of the animal. As with exteriorized cannula, the skill of the technician accessing, collecting samples, flushing, and locking the port directly affects the usable life of the port.

Urine Collection

Urine is generally collected in toxicology studies to assess kidney function. The most common method used for urine collection is a stainless steel commercially available cage. The cage is designed such that the urine and feces are separated by a cone-shaped device. The urine drains off the collecting walls into a tube and the feces fall into an inverted cone. Food and water are made available in such a way that the urine will not be contaminated. While this type of cage produces urine of acceptable quality for normal urinalysis, the sample may be contaminated with hair or feces. Other methods for urine collection in the rat include cystocentesis, which involves a needle stick into the bladder, and cannulation of the bladder.

Necropsy

The necropsy is the link between antemortem findings and histological observation. It is an essential portion of the toxicology study, and since a necropsy will involve the processing of a large number of animals, it is important that the procedure is well planned (Black, 1986). At a prenecropsy

meeting involving the pathologist, prosectors, and the study director, necropsy responsibilities can be discussed. Additionally, the study director can summarize clinical findings and potential target organs. The prosectors should be familiar with the protocol and amendments for the study involving the animals being necropsied. The protocol should clearly state which tissues are to be collected and weighed and how the tissue should be preserved. During the necropsy, devices such as checklists or prelabeled compartmentalized trays should be present to ensure that all required organs are taken and weighed. In recent years, commercially available computer software has been developed to assist in the collection and weighing of tissues at necropsy. This is an electronic copy of the checklist, but also provides a method for recording observations in a consistent manner. Copies of the last clinical observation should be present at the necropsy so the prosectors are alerted to lesions that may be present and require special attention. Palpation records are particularly important at carcinogenicity study necropsies to ensure that all masses detected at the last examination are confirmed and collected.

The necropsy will involve a checklist of animal identification and sex, an external examination of the animal, an in situ examination of all tissues and organs (prior to dissection), and the collecting and weighing of the required tissues.

Summary

In summary, there are several advantages to the use of the rat in toxicology studies. Because of its widespread use in many fields of biology, there is a large historical database of information about the anatomy and needs of the species. This knowledge, along with information about the species' metabolism and response to toxicants, has shown the rat to be generally a good model for the prediction of the human response to toxicants. Rats have a life span of 24–30 months, which is convenient for chronic toxicity and carcinogenicity studies where animals need to be exposed for the majority of their lifetime. The short gestation time and large litter size make the rat a good model for reproductive studies. The development of SPF rats and improvement in husbandry have eliminated most of the disease outbreaks that may have introduced variability into a study. The lack of an emetic response allows for the testing of higher dosages of compound that may cause vomiting in other species. The small size of the rat is useful in that a large number can be housed economically. The size is also useful in that smaller amounts of test compound are required to gain maximal exposure.

The relatively small size of the rat is also one of its major disadvantages. The amount of blood that can be taken from the animal is limited, thus limiting the number of parameters that can be investigated or the number of toxicokinetic samples that can be collected from a single animal. This problem can be overcome by adding additional animals in interim sacrifice groups or by collecting toxicokinetic samples from cohorts of animals at different times. In most cases, it is recommended that toxicokinetic samples be collected from satellite animals and not from the main study animals being used for the evaluation of toxicity. However, an increased number of animals mean increased work in the conduct of the study. The small size and relatively active nature of the rats make some procedures, such as intravenous dosing or collection of electrocardiograms, difficult. These issues have been overcome with the use of suitable restrainers or, in some case, anesthesia. The rat has been used successfully in toxicology research for close to a century and will continue to be used for the foreseeable future.

<div align="center">

PATHOLOGY

Shayne Cox Gad

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The rat continues to be the most popular species in toxicology, and the literature available on the toxicological pathology of the laboratory rat continues to outpace that of all other model species combined. It is thus not possible for this section to reproduce all of that literature. Rather, the desire

is to provide an overview of the key elements that most commonly influence the design, conduct, and interpretation of toxicity studies and then to give access to the relevant literature for those who need more information or detail. There are now a number of excellent texts addressing the toxicological pathology of the rat, and the interested reader is directed to them for a more in-depth review (Greaves, 2015; Haschek and Rousseaux, 1998; Haschek et al., 2002; Johnson et al., 2013).

Any discussion of rat toxicological histopathology must first start with differences due to age, strain, and sex. Our appreciation of the influence of these factors continues to evolve, particularly in the area of carcinogenesis (Haschek and Rousseaus, 2013; Johnson et al., 2013; Nakazawa et al., 2001; Percy and Barthold, 2007; Tennekes et al., 2004; Weber et al., 2011). It must always be kept in mind that the selection of strain (generally Sprague Dawley, Fischer 344, or Wistar, though others such as the Long-Evans do see use) is a feature of experimental design to be carefully considered. In the last year, the Wistar has become preferred over others (Taylor et al., 2012). Most contract research organizations (CROs) have a favorite strain, and all too frequently choice of the CRO leads to default choice of strain. Before the initiation of any study, there are three inputs from the laboratory animal professional and pathologist that must be considered for the optimal outcome of the study. First, the source of test animals must be carefully selected and all individual shipments carefully screened to ensure that only healthy rats with the desired characteristics (e.g., age, sex, strain) are used. This screening process should include a period of acclimation/health surveillance after the rats have been received to the facility (though 2 weeks is generally desirable, 7 days is adequate and currently more common).

Second, the objective of the study and the degree of variability in the pathology of the animals must be considered in the design of the study, as should any factors such as the known time course of a response (Haschek and Rousseaux, 2013; Percy and Burthold, 2007). Sufficient animals need to be used so that an effect may reasonably be detected (if present) as an increase above the existing background levels of morphological variation. In other words, the study design must be adequate to allow the development of a pathological response and yet also include "sampling" (collection of blood and tissues) at appropriate time points so that the effects of age or other confounding processes do not obscure results.

Finally, the necropsy must be designed to be both efficient and effective, and the involved personnel must have adequate training and experience with the type of animals being utilized.

Necropsy

Except for pilot or dose-ranging studies, unexpected mortalities in rodent toxicity studies should be limited to rare accidental deaths associated with procedures (dosing mistakes in intubation studies, for example) and deaths due to unexpected toxicity. Otherwise, animals should live to the time of scheduled termination, providing for optimal tissue collection and fixation and proper correlation of clinical signs, clinical chemistry, and pathology findings. Because moribund or dead animals frequently have treatment-induced or important spontaneous lesions, they must receive complete necropsies. Rats should be observed a minimum of twice daily to identify moribund individuals. If there is a significant chance that death may occur before the next observation, the animal should be sacrificed. Little is gained by attempting to have the animal live a few days longer, considering the risk of loss of data due to premature death and autolysis.

Dead animals should be refrigerated immediately on discovery and necropsied as soon as possible thereafter. Refrigeration will significantly delay autolysis (for up to 8 hours [Kupp and Strolle, 1979]). Dead animals should not be frozen, as ice crystals created during the freezing and thawing process will damage or destroy cellular integrity and severely limit or impair any interpretation of histopathology. For scheduled necropsies, animals should generally be fasted overnight to provide for the natural emptying of the GI tract and for a standard physiological baseline for the evaluation of clinical pathology parameters.

Clinical observation records of the animal should be available at the time of necropsy, enabling the prosector to focus on target sites of disease and decreasing the chance of missing important lesions. The necropsy technique may have to be modified to suit the clinical signs. For example, the standard necropsy calls for collecting a piece of lumbar spinal cord. If paralysis or paresis was noted clinically, the entire spinal cord and possibly the brachial or lumbar plexuses would need to be examined.

A standard necropsy procedure should be followed, as described in the separate chapter in this text. After reviewing the clinical records, one should palpate the animal thoroughly and examine all external orifices, eyes, and skin. Essentially, a physical examination is performed before the necropsy. The actual necropsy should follow a standardized format. This provides increased efficiency, optimal tissue accountability, and improved fixation. A thorough necropsy is probably the first essential step to assure eventual quality in pathology. General methods for rat necropsies and guides to gross anatomy have been published (Bohensky, 1986; Chiasson, 1988; Feldman and Seely, 1988; NCI, 1976; Reuber, 1977). If special considerations dictate alterations in which tissues are to be collected and are needed (usually seen as the addition of tissue sections or changes in preservatives used), these must be discussed thoroughly with the necropsy staff before initiation of the procedures.

Table 3.17 provides a list of the tissues that would be collected from a rat at necropsy, along with an indication of which should normally be weighed to provide an indication of potential morphological indications of treatment-related effects.

Organ weights can be extremely useful if accurately determined and when considered in the proper context. They can help to identify subcellular changes (such as enzyme induction in the liver) in target organs (though they are more useful for some organs than others; see Gad et al., 1984), and they can assist in identifying hormonal influences in some target organs. The downside is that improper tissue handling by the prosector can lead to artifactual tissue damage, and organ weights may not correlate with other findings (in which case, a weight of evidence and nature of the data warrant consideration).

If tissues, lesions, or observations are missed at necropsy, they will never be recovered.

Table 3.17   Tissues Collected and Weighed at Necropsy

Skin	Liver[a]
Mammary gland	Pancreas
Lymph node(s)	Spleen[a]
Salivary gland	Kidneys[a]
Sternum/costochondral junction	Adrenal gland[a]
Femur (including bone barrow)	Urinary bladder
Muscle (psoas)	Seminal vesicle
Thymus[a]	Prostate[a]
Trachea	Testes[a]
Lung[a]	Ovary[a]
Heart[a]	Uterus[a]
Thyroid gland (with parathyroid)[a]	Vagina
Tongue	Brain[a]
Esophagus	Pituitary gland[a]
Stomach	Spinal cord
Duodenum	Eyes
Jejunum	Lacrimal gland
Ileum	Any additional tissues or organs with "abnormal findings" or deemed appropriate
Colon	

[a] Tissues to be weighed at necropsy.

Accurate observations must be recorded and descriptions should include the location, size, color, and consistency of the lesion. Whenever possible, lesions should be collected and fixed with some adjacent normal tissue. Equipment for gross photography should be available to document significant study findings. Not all gross lesions must be photographed; however, representative toxic lesions should be recorded on film.

Routine tissues should be fixed in approximately 10 volumes of neutral buffered formalin. Excellent fixation of lungs can be obtained by inserting a 14- to 18-gauge blunt needle into the lower trachea and inflating the lungs to their original size with formalin. The stomach should be opened and examined carefully for ulcers, erosions, etc. The small intestine of rodents can be opened and examined or flushed with formalin and examined by transillumination. If the latter is done, the gut should be opened and examined at the time of tissue trimming. The bladder should also be inflated with formalin and examined by transillumination, followed by an open examination when trimmed for embedding. The pituitary is one tissue most often lost in rodent necropsies. This can be minimized by leaving the organ attached to the basisphenoid bone. The bone and attached pituitary can be placed in a special tissue capsule until embedding. After removal of the eyes, brain, pituitary, tongue, and mandible, the nasal turbinates can be fixed by flushing from the nasopharynx to the nares with formalin followed by immersion in formalin. Tissue sections should have a maximum thickness of 0.5 cm for good fixation and should be fixed for a minimum of 48 hours before trimming and processing.

Clinical Pathology

Clinical pathology comprises both hematology and clinical chemistry. These are extremely powerful tools for both assessing specific target organ toxicities during the in-life phase and providing correlative information for understanding both disease processes and the relevance of anatomical findings.

Laboratory determination of various clinical chemistry parameters in the rat is not particularly difficult, especially with the recent development of many automated analyzers. Most of these instruments require from 1.5 to 50.0 μL of serum or plasma and are preprogrammed to perform up to 20 different clinical chemical tests. The primary challenge of the clinical pathology laboratory is to validate equipment or procedures designed for use with human serum, or blood, and modify these into reliable methodology for the evaluation of organ function and toxicity in rats. Toxicologists must be aware of a seemingly infinite number of variables that impact upon the interpretation of clinical pathology. Controllable sources of variation can be divided into at least three general categories: (1) variation related to the physiological status of the animal and its environmental condition, (2) variation related to sampling, and (3) variation related to analytical instrumentation and methodology.

Several authors have addressed the effect of these factors on many of the common clinical chemistry procedures. Variations related to the physiological status of the rat and its environment include disease status (Cotchin and Roe, 1967; Deb and Hart, 1956), age (Kozma et al., 1969; Vondruska and Greco, 1973; Weisse et al., 1974), sex (Kozma, 1969; Weisse et al., 1974), husbandry, nutritional condition, and degree of hydration. Variations related to sampling collection include method of collection, anesthetic used, the time of day the sample is collected, restraint technique, anticoagulant used, hemolysis, sample processing and storage, and site of sampling. Blood sampling techniques are described and discussed elsewhere in this chapter.

In the rat as in other species, prompt separation of serum from cells is critical. Hemolyzed or transparent serum that has remained in contact with erythrocytes can have falsely elevated values for potassium, lactic dehydrogenase (LDH), and total protein, less consistently and to a lesser degree for phosphorus and sometimes for bilirubin. Prolonged contact of serum with erythrocytes

reduces serum glucose. Centrifugation and prompt removal of a serum from the clot should occur at intervals not exceeding 30 min after collection in order to yield reliable data.

Tables 3.18 and 3.19 summarize literature values for clinical chemistries and hematological values of common laboratory rats. One notable variation in hematologies for different strains of rats is the life span of red blood cells (Derelanko, 1987), which suggests that other functional aspects of the hematological system may vary between strains to the point that they can influence interpretation. All such tabular summaries of normal or control values (such as Leonard and Ruben, 1986; Mitruka and Rawnsley, 1977; and Loeb and Quimby, 1989), however, should be considered critically and used only as general guidance. Current control values for a particular strain and CRO are generally much more meaningful for comparison than literature values.

Table 3.18   Clinical Chemistry Values

	Males		Females		
	Mean	SD	Mean	SD	Range
Bilirubin (mg/dL)	0.35	0.02	0.24	0.07	0.00–0.55
Cholesterol (mg/dL)	28.3	10.2	24.7	9.62	10–54
Creatinine (mg/dL)	0.46	0.13	0.49	0.12	0.20–0.80
Glucose (mg/dL)	78.0	14	71	16	50–135
Urea nitrogen (mg/dL)	15.5	4.44	13.8	4.15	5–29
Uric acid (mg/dL)	1.99	0.25	1.79	0.24	1.20–7.5
Sodium (mEq/l)	147	2.65	146	2.50	143–156
Potassium (mEq/l)	5.82	0.11	6.70	0.12	5.40–7
Chloride (mEq/l)	102	0.85	101	0.90	100–110
Bicarbonate (mEq/l)	24	3.80	20.8	3.60	12.6–32
Phosphorous (mg/dL)	7.56	1.51	8.26	1.14	3.11–11
Calcium (mg/dL)	12.2	0.75	10.6	0.89	7.2–13.9
Magnesium (mg/dL)	3.12	0.41	2.60	0.21	16.44
Amylase (Somogyi units/dL)	245	32	196	34	128–313
Alkaline phosphatase (IU/l)	81.4	14.8	93.9	17.3	56.8–128
Acid phosphatase GIULIA	39	4.30	37.5	3.70	28.9–47.6
Alanine transaminase(SGPT) (IU/l)	25.2	2.05	22.5	2.50	1.5–30. 2
Aspartate transaminase (SGOT) (IU/l)	62.5	8.40	64.0	6.50	45.7–80.8
Creatine phosphokinase (IU/l)	5.60	1.30	6.80	2.40	0.84–11.6
Lactic dehydrogenase (LDH) (IU/l)	92.5	13.9	90	14.5	61.0–121
Serum total protein (g/dl)	7.61	0.50	7.52	0.32	4.70–8.15
Albumin (g/dl)	3.73	0.53	3.62	0.52	2.70–5.10
(%)	49	7.10	48.1	7.40	33.3–63.8
α_1-Globulin (g/dl)	1.03	0.22	0.89	0.25	0.39–1.60
(%)	13.5	2.20	11.9	3.80	4.30–21.1
α_2-Globulin (g/dl)	0.71	0.14	1.40	0.32	0.20–2.10
(%)	9.3	1.80	8.60	2.70	3.20–14.7
β-Globulin (g/dl)	1.07	0.35	1.31	0.26	0.35–2.00
(%)	14.1	4.70	17.4	3.60	5.70–26.8
γ-Globulin (g/dl)	1.05	0.21	1.18	0.21	0.62–1.60
(%)	13.8	2.70	14	2.80	10–19.8
Albumin/globulin	0.96	0.24	0.93	0.25	0.72–1.21

Table 3.19 Hematology Values of Common Rat Strains

Test	Units	Long-Evans (Blu:LE)	Wistar/Lewis Albino	Osborne-Mendel	Fischer Inbred Strain 344/Cr
Erythrocytes (RBC)	$(\times 10^6/mm^3)$	5.98–8.30	7.20–9.60	36.26–8.96	6.68–9.15
Hemoglobin	(g/dL)	13.1–16.7	12–17.5	14.30–17.7	13.4–17.2
MCV	(v^3)	52–69	57–65	52–66	54–67.5
MCH	(vvg)	18.5–23.5	14.6–21.3	18.8–23.3	17–21.8
MCHC	(%)	32–38.5	26–38	32–42	26–35.5
Hematocrit (PCV)	(mL%)	39–48	42.5–49.4	39.4–46.2	46–52.5
Leukocyte (WBC)	$(\times 10^3/mm^3)$	3.30–7.90	5–8.96	6.23–12.6	5.35–11.2
Neutrophils	(%)	5.50–35.5	9–34	4.50–23.5	11.5–41.6
Basophils	(%)	0	0–1.50	0	0
Lymphocytes	(%)	60–93.5	65–84.5	72–94	43–79.5
Monocytes	(%)	0–5.50	0–5	0.50–3.50	0–2
Eosinophils	(%)	0–1.50	0–2.50	0–1	0–4
Platelets	$(\times 10^3/mm^3)$	140–460	160–470	145–450	150–450

Common Diseases

Common diseases that occur in modern caesarian-derived (CD), SPF rats used in toxicology laboratories can be viral, bacterial, parasitic, or rickettsial. Outbreaks of infectious disease are becoming less common in well-managed laboratory animal facilities, but sporadic outbreaks and subclinical infections do occur. It behooves the laboratory animal professional to maintain high operating standards to minimize the impact of infectious diseases in toxicity studies. Infectious diseases can produce lesions and functional defects that may be difficult to distinguish from those of target organ toxicity. Infectious agents may produce lesions at the site of entry equivalent to local toxicity, and lesions equivalent to systemic toxicity. Either local or systemic effects can predominate, or the disease may be expressed as a combination of both. Infectious skin diseases are uncommon in most laboratory animals, and the two most frequently affected portals of entry are the respiratory and digestive tracts. In rats, infections of the respiratory tract are more frequent.

Viral Pneumonitis

Viral pneumonitis is a subclinical respiratory infection producing a lung lesion that is a focal alveolitis, which may be observed in any phase of the inflammatory process from acute to chronic and is accompanied by prominent perivascular leukocyte foci, usually lymphoid, with occasional eosinophils. The alveolitis is generally of little clinical significance, but its presence can seriously confound the interpretation of inhalation toxicity studies, particularly of some of the low-grade focal dust-induced lesions. Hyperplasia of the mandibular lymph node is also common in infected rats and probably represents an immune reaction to viral infection. The causal organism may be the pneumonia virus of mice (PVM).

Sialodacryoadenitis

Sialodacryoadenitis (SDA) is a frequent epizootic disease in rat colonies with a high morbidity and negligible mortality. It was first recognized in rats in 1961 (Innes and Stanton, 1961). SDA is a generic term for infection with several serotypes of a coronavirus. These serotypes have a spectrum of virulence, primarily infecting the upper respiratory tract with variable infection of the glands around the head and neck.

Respiratory tract lesions include rhinitis and focal interstitial pneumonitis, but this is generally subclinical. In contrast, infection of the glands often produces striking clinical symptoms. The most striking clinical features are red staining around the eyes due to infection of the Harderian gland and swelling of the ventral neck region associated with infection of the submaxillary salivary gland. The swollen neck ("rat mumps") rapidly subsides, and the rats appear normal within about a week. Thus, SDA is a disease dominated by acute local effects.

The main finding at necropsy, depending on the stage of the disease, is either a swollen or shrunken salivary gland. Histologically, the gland progressively shows various combinations of degeneration, inflammation, and regeneration, but it is quickly restored to normal. The Harderian gland shows a similar cycle of disease, but squamous metaplasia is very prominent in the proliferative repair phase. In some animals, there are ophthalmic lesions, including reddish discharge, cloudy cornea, and corneal ulceration. Microscopically, the changes in the Harderian and submaxillary salivary glands can be dramatic: there is necrosis, intense inflammation, and often marked squamous metaplasia of the ductal epithelium.

Lesions in the eye associated with SDA consist of keratoconjunctivitis, corneal ulcers, and synechiae of the iris and ciliary body and are largely resolved by 10 weeks; however, megaloglobus persisted in 6% of the rats in one study (Lai et al., 1978). Such severe alterations of ocular structures make infected rats unsuitable for research in which the eye is the target organ. Apparently, this disease is widespread in rat colonies, invading the ear canal, and usually has both sebaceous and squamous differentiation.

Sendai Virus Infection

The Sendai virus is a paramyxovirus that is enzootic in many modern rat colonies. Enzootic infections generally affect weanlings as maternal milk-transmitted immunity wanes. The pups develop a respiratory disease that is generally subclinical. At necropsy, many rats appear normal, but some have small red foci scattered over the surface of the lung. Histologically, there are acute necrotizing inflammatory lesions in the mucosal epithelium of the nose, trachea, and pulmonary airways, the last of these often extending into the alveoli. Most of the airway mucosal lesions repair rapidly and completely, but focal fibrosis and scarring may occur in terminal airways and associated alveoli. In general, however, Sendai infection can be considered an acute transient infection of the rat respiratory tract with little or no residual effects.

Although it is widely recognized that Sendai virus is a respiratory pathogen of mice that produces pneumonia and death, its role in naturally occurring respiratory diseases of rats is much less clear. In one report, a Sendai virus epidemic occurred in an aging rat colony paralleling an outbreak in a mouse colony at the same institute (Burek et al., 1977). The epidemic was characterized by seroconversion, but increased mortality and clinical signs were not observed. During this period, however, rats coming to necropsy had distinct microscopic pulmonary lesions not found before the outbreak. Three types of lesions were found: perivascular cuffing of pulmonary vessels by plasma cells and lymphocytes; multifocal interstitial pulmonary infiltrates of macrophages, lymphocytes, and neutrophils; and hyperplasia of peribronchial lymphoid tissue.

Corynebacterium kutscheri *Infection*

Corynebacterium kutscheri is an example of a bacterial disease dominated mainly by acute local effects in the respiratory tract. The infection is usually latent or inapparent, but may become activated to produce acute suppurative pulmonary lesions that either are fatal or resolve by fibrosis and formation of granulomas. Activation of this disease is usually associated with factors that lower host resistance. Administration of chemicals could be such a factor, through either immunosuppression or the general stress of toxicity. As a result, a pathologist could be presented with a pattern of

minor lesions in controls and a dose-related increase in lung lesions in treated groups. This apparent "pulmonary toxicity" is another example of the complex interaction between different causal agents that may occur in animal models.

Pinworms

Pinworms are parasitic nematodes that may be very common and are visibly living free in the lumen of the large intestine without producing any obvious lesions. They are common in many species and are a good example of an asymptomatic local infection.

Mycoplasmosis

Mycoplasmosis tends to be tissue and host specific, and in the rat, the disease primarily affects the respiratory tract and to a lesser extent the female reproductive tract. The respiratory disease begins in early life and progresses as a chronic condition; therefore, respiratory mycoplasmosis in the rat is commonly termed chronic respiratory disease (CRD). CRD has been the major health problem affecting the laboratory rat. When endemic in a colony, it is generally the life-limiting factor. This disease has been eliminated from most modern colonies, but is still occasionally encountered as a highly contagious, chronic enzootic disease of the respiratory tract.

Mycoplasmosis often begins with inner ear involvement, and only later is pulmonary involvement seen (Kohn, 1971). The most characteristic clinical sign in the young rat is snuffling and wheezing during the first 3 months of life. This snuffing is associated with inflammation in the upper respiratory tract, notably rhinitis, and with increasing severity or age progressively affects the larynx, trachea, and lungs to produce the bronchiectatic abscess characteristic of the older rat. After mycoplasmal colonization, small amounts of purulent exudate appear in the airway lumen. This is followed by hyperplasia and increased mucus production in the respiratory epithelium and by lymphoid infiltration and proliferation. The hallmarks of chronic infection are thus exudate, epithelial hyperplasia, squamous metaplasia, and lymphoid cell accumulation. The infection may also affect the middle ear, and subsequent labyrinthitis may produce clinical symptoms such as circling.

Like *C. kutscheri* infection, the disease pattern may be altered by extraneous factors such as ammonia levels in the animal room as well as by interaction with other infectious agents such as the Sendai virus, and of course by effects related to test article administration. Any stress such as crowding or experimental procedure will cause increased mortality and decreased life span.

Gross pathological findings include areas of red to gray consolidation of the lung, often containing abscesses. In a majority of rats, the bullae of the ears contain a greenish-yellow exudate. The most spectacular pathological changes are seen in the old rat, and in protracted cases, the lung may be converted to a mass of bronchiectatic tissue and abscesses. Microscopically, there is a severe chronic purulent bronchopneumonia with bronchiectasis and marked peribronchial lymphoid hyperplasia. The histopathological findings in advance stages are fairly typical or chronic disease, consisting of a combination of chronic inflammation and reparative processes.

While numerous agents have been proposed as the cause of CRD, *Mycoplasma pulmonis* appears to be the main, and in many cases the sole, agent responsible. The organism can continue to be isolated from the pneumonic lung, and rats with more severe respiratory involvement are more infectious for other rats. Although high complement fixation antibody titers are present and may serve as an aid in diagnosis, there is no correlation between circulating antibody and the presence of the organism. This suggests that circulating antibody does not play a role in protection against the mycoplasma infection. Occasionally, *Mycoplasma arthritidis* can be a secondary invader in CRD of rats. Common nonneoplastic lesions are summarized in Table 3.20, while changes in incidences in age at termination are presented in Table 3.21.

Table 3.20 Example of a Pattern of Pathology in Young Rats

Organ	Diagnosis	% of Incidence	
		Males	Females
Skin	Alopecia/fur loss	5	9
	Dermatitis/sore	4	4
Tail	Dermatitis/sore	5	3
	Other lesions	3	3
Esophagus	Myositis	2	6
Eye	Periorbititis	10	11
	Other lesions	6	8
Heart	Leukocyte foci	7	2
Kidney	Leukocyte foci	12	7
	Hyaline droplets	15	0
	Tubular regeneration	32	4
	Mineralization	0	6
	Hydronephrosis	10	10
Liver	Leukocyte foci	80	80
Lung	Leukocyte foci	52	56
	Pneumonitis	20	18
	Foamy histiocytes	20	16
	Mandibular lymph node hyperplasia	40	26
	Large intestine nematodes	8	3
Pituitary	Cyst	3	3
Stomach	Erosions and ulcerations	10	10[a]
Thyroid	Ectopic thymus	2	3
Prostate	Leukocyte foci	14	—
Testis	Atrophy	2	—[b]
Thymus	Atrophy	—	—[b]
Uterus	Distension	—	14

Source: Adapted from Glaister, J., *Principles of Toxicological Pathology*, Taylor & Francis Group, Philadelphia, PA, 1986.

[a] Seems to be increasing in recent years (1988 onward).
[b] Commonly found associated with nonspecific stress at higher dose levels.

Histopathology of the Rat

Though there are several ways that one might organize an overview of the microscopic pathology of the laboratory rat, a good (and fairly common) approach is to compare the patterns commonly found in both young and aged animals, as these form very different backgrounds to evaluate (or detect) any toxicological response against secondary patterns of concern, which may complicate or confound determinations of causality, such as a response to generalized stress that is not uncommonly seen at high doses may then also be considered. In all cases, refer to some standard glossary for use in the description of findings (such as that of Greaves and Faccini, 1992).

Pathology of Young Rats

Strain-to-strain variations for patterns of age-related lesions are recognized in rats. Though the patterns associated with old animals are generally of more interest, there are also common spontaneous lesions in young animals. Control rats rarely die in short-term studies and neoplasia is also rare, so significant treatment group–related incidence of tumor after 6 months or less of treatment

Table 3.21   Incidence (%) of Morbidity and Mortality in Sprague Dawley Rats Due to the Major Groups of Nonneoplastic and Neoplastic Causes

| | Study Internal (Weeks) | | | | | |
| | Males | | | Females | | |
Cause	52–78	79–104	>104	52–78	79–104	>104
Nonneoplasia						
Degeneration	8	8	12	2	1	0
Inflammation	8	8	8	2	2	2
Other conditions	2	2	8	1	1	5
Uncertain/multifactorial	14	19	8	2	2	0
Neoplasia						
Pituitary	40	30	21	45	41	45
Subcutis	19	13	16	1	2	2
Mammary	0	0	0	33	43	32
Other tumors	9	20	27	14	8	14

Source: Adapted from Glaister, J., *Principles of Toxicological Pathology*, Taylor & Francis Group, Philadelphia, PA, 1986. Coleman et al. (1977) have extensive incidence tables on male Fischer 344 rats.

is very striking. The observed pattern of pathology is, therefore, of nonneoplastic lesions and is usually presented in tabular form, such as in Table 3.20. Only lesions occurring with an incidence greater than 1% are listed. A toxicologist presented with such a table should try to understand what the diagnoses and their frequencies mean in terms of the general background of pathology in control animals. Among the 40 organs commonly evaluated, only a minority has lesions, and relatively few lesions have incidence rates greater than 10%. Thus, the majority of organs of a young rat are either normal or rarely show histopathological changes. There are numerous subpatterns within this general overall pattern of infrequent lesions. The observations presented in incidence summary tables, such as Table 3.20, are usually listed on an organ-by-organ basis, but lesions within any particular organ may represent degenerations, inflammations, or proliferations; they may be trivial or severe; and they may be related to a variety of causes. It is the association between cause and effect that is the main interest in toxicological pathology, and in young animals, it is convenient to describe the pathology in terms of causation rather than in terms of lesions within any one organ.

Rats are commonly housed in small groups, often in metal cages with wire mesh floors. A variety of minor external inflammatory lesions are encountered owing to attrition between rats and between rats and their cages. Fur loss and dermatitis are typical changes seen on the skin and on the tail in group-housed rats. Lesions on the feet and ears may also be seen. These lesions are usually dismissed as insignificant, but differences between control and treated groups may occur in certain circumstances, such as a decrease in the incidence of fight wounds in rats given tranquilizers.

Some lesions are associated with the method of administration of the chemical or with the removal of blood samples for analysis. Rats may struggle when gavaged and the esophagus can be traumatized by the cannula. This bruising appears histologically as a focal myositis with evidence of acute inflammation and/or with healing and repair. The usual appearance is one of basophilic regenerating myofibers accompanied by a few leukocytes. Although the oral route is the most common method of test article administration, equivalent inflammatory lesions such as phlebitis or myositis may be common following intravenous and intramuscular administration. It should always be kept in mind that the point of introduction of a drug or test substance into the body (such as the vasculature immediately in the region of a catheter) may have some unique pathology associated with experiencing the highest concentration of an administered agent. This pathology may be more associated with physiochemical factors than with actual drug toxicity. This holds true for any parenterally, nasally, or topically administered agents, and sometimes for agents administered orally.

Hair Fragments in the Lung

Another lesion sometimes associated with intravenous injections is hair fragments in the lung. These fragments lodge in pulmonary vessels as hair emboli after their introduction into a peripheral vein during venipuncture. Blood sampling by orbital sinus puncture is another common procedure, and this may cause a variety of inflammatory changes around the eye and orbit, designated by the general term periorbititis. Repeated sampling or poor technique may produce other lesions such as degeneration of the optic nerve (optic neuropathy). The severity and incidence of these procedure-related lesions are variable depending on the nature and frequency of the procedure and on the skill of the technician. Lesions may be uncommon and minor in short-term, well-conducted studies, or at the other extreme may be fatal if the esophagus or eyeball is accidentally punctured.

Congenital or Developmental Defects

The thymus has the same embryological origin as the parathyroid, and during its migration toward the base of the heart, small nests of tissue descending from the third and fourth bronchial pouch may remain adjacent to or be embedded in the parathyroid/thyroid. These may be quite striking histologically, but they are of trivial significance. Similarly, pituitary cysts are usually trivial. They are derived from remnants of the upgrowth of the craniopharyngeal (Rathke's) pouch, which develops into the adenohypophysis. They vary in site and appearance, but they are usually seen in the pars distalis as small colloid-filled cysts lined by ciliated epithelium. Hydronephrosis (dilation of the renal pelvis) may be caused by urinary tract obstruction, but more commonly is usually due to developmental or congenital defects. It tends to be unilateral, of little significance, and is associated with normal renal function. Severe bilateral and fatal cases may occasionally occur, but care must be taken to exclude obstruction of the lower urinary tract in these instances.

Background Changes

A variety of minor inflammations, degenerations, and proliferations of diverse or uncertain cause may be encountered in the young rat. They are grouped together as miscellaneous "background" changes and include (see Table 3.20) hyaline droplets, tubular regeneration and mineralization in the kidney, foamy histiocytes in the lung, testicular atrophy, and uterine distension. One also sees leukocyte foci in the liver and lung.

Renal Hyaline Droplets

Dense eosinophilic "hyaline" droplets of variable size are seen in the cytoplasm of proximal tubular cells in both man and experimental animals. The kidney is not only the major route of elimination for many agents but also an important site of uptake and catabolism of low-molecular-weight proteins such as albumin, immunoglobulin light chains, parathyroid hormone, and glucagons. The main pathway for the extraction of proteins from the circulation is by glomerular filtration. The amount of protein reaching the urinary space is dependent on glomerular filtration rate, plasma concentration, and the physicochemical characteristics of the protein, and the degree of uptake is inversely proportional to the molecular size of the protein. The majority of protein within the tubular fluid is taken up into the proximal tubule by endocytosis. Absorbed proteins within endocytotic vacuoles are transported to regions of the tubular cell rich in lysosomes where fusion takes place and hydrolysis to amino acids occurs. Amino acids are then returned to the circulation. Disturbance of this balance, by either increased filtered loads of proteins or their decreased catabolism may result in the accumulation of protein in the form of "hyaline" droplets in renal tubular lysosomes.

Hyaline droplets are particularly prominent in the proximal renal tubules of untreated male rats, and male rats are liable to show increased droplet formation after treatment with a wide range of drugs and chemicals. This predisposition appears to be due mainly to the presence of a specific protein, α_{2u} globulin, which is the major normal urinary protein in the male rat, synthesized by the liver, and under synergistic control by testosterone and corticosterone (Alden, 1985). Although humans excrete proteins of a similar nature, they are found in only trace amounts (Hard, 1995). This protein has a molecular weight of 18,000–20,000 Da, and it is freely filtered by the glomerulus and reabsorbed by the proximal tubular cell. Immunocytochemical study has shown its location to mainly in the S2 segment of the proximal tubule in the male rat. Moreover, the male rat may have a lower capacity of renal protein handling than females because protein tracer studies using ovalbumin have shown that the male rat proximal tubule has a lower rate of reabsorption and catabolism of protein ultrafiltrate than female mice.

It should be noted that other proteins are also linked to the formation of hyaline droplets spontaneously in rodents. One example is the increase in hyaline droplets reported in rats and mice suffering from histiocytic sarcoma that immunocytochemistry showed contained lysozyme but not increased amounts of α_{2u} globulin or other proteins such as α_1 antitrypsin or immunoglobulin (Hard and Snowden, 1991).

Similar morphological changes may be observed in the male rat following the administration of pharmaceutical agents and may also be the result of binding of drug with lysosomal protein constituents in the proximal tubule, which impairs their catabolism. This mechanism was suggested to explain the hyaline droplet accumulation found in the S1 and S2 segments of proximal tubules in male rats treated with the antihelminthic, levamisol, the antimalarial BW 58c, and an anti-inflammatory 540c. These histopathological features were different from the myeloid bodies found in the renal tubules following treatment with aminoglycosides and other lysosomotropic agents.

Small cytoplasmic droplets reflecting the accumulation of dense secondary lysosomes are reported in the cytoplasm collected from the renal papilla rats treated with carbonic anhydrase inhibitors (Owen et al., 1993, 1994). As these effects were ameliorated by administration of potassium, it was argued that their development may be related to depletion of renal medullary potassium.

While injection or infusion of normal proteins may simply increase the number of hyaline droplets with identical morphology to those found spontaneously, it should be kept in mind that even normal proteins have differing nephrotoxic potential. For instance, perfusion of rats with human immunoglobulin k light chain of molecular weight of between 20,000 and 50,000 Da was associated with droplet formation and acute tubular injury, whereas albumin was devoid of nephrotoxicity under similar circumstances. Although the reason for this difference is unclear, physicochemical properties are presumably important. It is of interest to note that k immunoglobulin light chains are also capable of inducing acute tubular injury in humans.

Renal hyaline droplets are common findings in the kidneys of the male rat. These appear as bright eosinophilic globules of various sizes in the cytoplasm of the proximal tubular epithelium. The glomerulus of the male rat is "leaky" to low-molecular-weight proteins, and these droplets represent protein resorbed by the proximal tubule cells and sequestered in lysosomes prior to recycling into the blood. Although these droplets are common and are generally considered trivial, like many other background observations, the severity of the change occasionally increases in a dose-related manner. The cause of such an increase in hyaline droplets is related to the interaction of the test article or a metabolite with a protein, α_2 microglobulin, and interference with the recycling of the globulin by renal tubules. In some cases, the increase in hyaline droplets is accompanied by focal tubular degeneration and/or tubular necrosis. Thus, a common change may be accentuated to a sufficient extent in treated animals to be variably interpreted as evidence of nephrotoxicity. The kidneys of females are usually normal.

Renal Tubular Regeneration

Renal tubular regeneration is another common but minor finding in male rat kidneys. Synonyms are "blue tubules," tubular atrophy, and tubular basophilia. The affected tubules stand out against the generally eosinophilic cortex because of their basophilia. Upon closer inspection, they appear as slightly shrunken tubules with cuboidal basophilic epithelium and sometimes a thickened basement membrane. The response of tissues to injury is limited, and tubular regeneration is one sequel to nephrotoxicity. This otherwise trivial lesion may therefore assume importance if it confounds the interpretation of no-effect levels in studies of nephrotoxic chemicals.

Renal Tubular Mineralization

Renal mineralization, also termed nephrocalcinosis, is a common minor renal lesion, but is seen mainly in females. The mineral may be found under or in the pelvic epithelium, or more commonly in the tubules at the corticomedullary junction. The corticomedullary mineralization usually appears between weaning and sexual maturity, and the etiology appears to be multifactorial, involving dietary and endocrine factors. Manipulation of dietary components in nutritional studies on certain food or protein substitutes may increase the incidence and severity of mineralization. This is another example of synergism between the specific substance of interest and background factors, and it may be difficult to establish no-effect levels if there is a high incidence of mineralization in controls.

Foamy Histiocytes

In the lung, the main observation of interest is foamy histiocytes. These are also called foam cells or termed lipidosis. The condition is characterized by accumulations of plump pale cells in the alveolar lumen, often located subpleurally so that they appear at necropsy as pinpoint gray spots on the surface of the lung. The foamy appearance of these alveolar macrophages is due to the uptake of surfactant released from type II alveolar cells. This condition may be considered an uptake, storage, and recycling process analogous to the hyaline droplets seen in the male rat kidney and, similarly, may become enhanced in response to certain treatments. This enhancement is seen most commonly in response to inhaled particles such as silica and in association with phospholipidosis-inducing compounds such as cationic amphiphilic drugs. A high control incidence of foam cells may interfere with the determination of no-effect levels with these compounds.

Testicular Atrophy

Testicular atrophy is usually minor and unilateral and affects the subcapsular tubules. Occasionally, it may be severe and diffuse and appears at necropsy as a small, watery, blue testis. Histologically, the affected tubules are small and show partial or complete lack of germ cells, leaving only Sertoli cells. This condition can have many causes, including congenital origin, obstructive lesions, and physical restraint as in nose-only inhalation studies. It may also result from treatment with chemicals and hormones, and a high background incidence in controls may hinder the evaluation of these substances. It is not uncommon to see significant incidences of testicular and thymic atrophy in high-dose group animals attributable to nonspecific stress rather than any specific target organ toxicity. In these cases, however, the animals also generally have reduced food consumption and reduced absolute body weight.

Leukocyte Inflammatory Cell Foci in the Liver and Lung

Leukocyte inflammatory cell foci are frequently encountered in certain tissues, especially the liver and lung and to a lesser extent the prostate. They may be related to enzootic viral infections

such as PVM or Sendai, but the relationship is difficult to establish. The foci are usually minor and multifocal, and usually comprise mononuclear leukocytes in various admixtures, but mainly lymphoid. These foci appear histologically as small basophilic cellular aggregates scattered across the plane of section. Other terms used are round cell foci, lymphoid foci, inflammatory foci, and similar nonspecific terms. This tendency to use nonspecific terms is quite common among toxicological pathologists because of the frequent misunderstanding of the more specific terms when crossing species' lines or scientific disciplines. Thus, leukocytes in the liver may occasionally surround a necrotic liver cell and could justify the term hepatitis. However, such a diagnosis may be interpreted as a serious condition by a medical pathologist or a toxicologist unfamiliar with laboratory animal pathology.

In terms of the two basic scales for accession lesions—incidence and severity pathology—findings in the young rat can be considered to be generally infrequent and, when present, of minor biological significance. Their main importance is in the two ways in which they can complicate the interpretation of treatment-related responses. In the first place, they may mimic the effect of treatment. For example, renal tubular regeneration may complicate the assessment of no-effect levels of nephrotoxins. Second, the incidence or severity of background pathology may be enhanced in a dose-related manner. The enhancement may be of endogenous processes such as the lysosomal cycling of pulmonary surfactant and renal protein, or it may be of processes initiated by exogenous agents such as infections. In either case, the precise causal relationship of treatment to the observed response may be difficult to unravel.

Pathology of Old Rats

The pathology of the aged rat is an important confounding factor in long-term bioassays for chronic toxicity and carcinogenicity (see Chandra and Frith, 1992; Nakazawa et al., 2001). In contrast to the pattern of infrequent and generally minor lesions seen in young rats, the pattern seen in aging animals during chronic studies is one of an increasing incidence and severity of lesions culminating in death. Although lesions related to husbandry, procedures, development, and infections still occur, these are relatively minor compared with the main age-associated lesions and, therefore, the pattern of pathology will be classified in a different way. The three main items of interest in chronic rodent studies are morbidity/mortality, nonneoplastic lesions, and neoplasms. These will vary from strain to strain (more so than the patterns associated with very young animals), but a basic pattern is still present. The two main parameters of interest in analysis of morbidity (illness) and mortality (death) are the rate at which they occur and the causation. These data give the pathologist and toxicologist an excellent opportunity to overview some of the major background characteristics of the rat strain. One or both of these parameters may be affected by treatment, but must also always be considered in study design and conduct. The status of changes in body weight (are the animals gaining, losing, or stable) is an important indicator of general health status for a group of rats.

Age at Which to Terminate Animals

A key issue in rodent carcinogenicity studies is the age at which to terminate the animals. As such a study progresses, the rise in the background level of tumors makes it more and more difficult to clearly partition treatment-effect tumors from age-effect tumors. Swenberg (Solleveld et al., 1984; Swenberg, 1985) has made the point that the incidence of many tumor types has increased from 100% to 500% when control rat results from 2-year studies (rats 110–116 weeks of age) were compared to those from life span studies (140–146 weeks of age). If such an increase in age (25%) can result in such extreme increases in spontaneous tumors, what is the effect on

interpretation of incidence rates seen in concurrent treatment groups? This is especially the case if, as Salsburg (1980) has suggested, any biologically active treatment will result in a shift in the patterns of neoplastic lesions occurring in aging animals. The current practice is to interpret tumor incidence on an independent site-by-site basis (on the assumption that what happens at each tissue site is independent of what happens elsewhere), and no allowance or factoring is made for the fact that what may be occurring in animals over their life span (as expressed by tumor incidence levels at an advanced age) is merely a shifting of patterns from one tumor site to another. In other words, commonly the "significantly" increased incidence of liver tumors is focused on by reviewers and authors, whereas the just as statistically significant decrease in kidney tumors compared to controls is ignored. Clearly, we should not be trying to analyze tumor data from rats that are advancing into senescence in the same manner that we do for the data from those that lack these confounding factors. Where should a cutoff point be? This is a problem, but clearly Cameron's data (Cameron et al., 1985) suggest that the growth curves of 9385 B6C3F1 mice and 10,023 F344 rats from control groups in NCI/NTP studies show consistent patterns of decline in body weights from these animals starting at the following ages (in weeks):

	Males	Females
B6C3F1 mice	96	101
Fischer 344 rats	91	106
Sprague Dawley rats	112	104

The existence of similar data for tumor incidences (unfortunately not available from NC1/NTP studies) would certainly improve our confidence in selecting cutoff points for age, but these ages merit consideration as termination points.

Patterns of Lesions

The patterns of lesions are equally as important as the rates and incidences at which they occur, as these can also be altered by treatment and may provide essential insight into the causal relationship. Such patterns can be altered without any change in survival rates.

In rats, the patterns of neoplasia associated with deaths on carcinogenicity studies are sex specific. Approximately 80% of unscheduled deaths in females are attributable to tumors in two organs, the pituitary and mammary gland, and this pattern is constant throughout the aging period. Other lethal tumors considered on an individual organ basis account for less than 5% of morbidity and mortality, and a similar case holds for various types of nonneoplastic lesions. Pituitary tumors cause illness and death because of their critical position at the base of the brain, and relatively small tumors may be fatal. Mammary tumors are not critically located. These subcutaneous tumors may exceed 100 g without causing any apparent ill health unless they ulcerate or impede the animal's movement.

The patterns of mortality associated with neoplastic lesions in males are more diverse. Two main tumor types, pituitary tumors and fibrous tumors of the subcutis, account for many losses, but the pattern is not as prevalent in females, in which several other tumor types occur. The incidence of these other lethal tumors tends to increase with time, but rarely exceeds 5% if they are considered on an individual tissue basis. Two tumors predominate, liver tumors and various endocrine tumors, and are accompanied with time by a slowly increasing range of low-frequency tumor types such as bone tumors.

About one-third of unscheduled necropsies in males are not associated with any large, ulcerated, or critically situated neoplasms, and morbidity and mortality in these cases have to be attributed to nonneoplastic causes. In many cases, there may be no major morphological lesion to account for

illness, or there may be two or more conditions of equivalent biological impact, and the cause has to be stated as uncertain or multifactorial. In other cases, there may be major degenerative lesions in the kidney, nervous system, or heart or inflammation in the skin, appendages, or genitourinary tract, and these together with other obvious lesions can be attributed unequivocally as the cause of the animal's demise. The pattern of these nonneoplastic causes of death has a reasonable constancy with time, except that the uncertainty tends to decrease as the major degenerations associated with old age become more prominent. In some strains, particularly those fed high-protein diets ad libitum, kidney disease can be the major cause of morbidity and mortality in males.

Analysis of the incidence and causes of morbidity and mortality is not a common practice in long-term studies, but has much to commend it in assessing the biological significance of lesions. Changes in mortality rate or in the major patterns of causation are easily detected and are more likely to be relevant in risk assessment than any minor increases in the incidence of microscopic or small tumors found in groups of animals dying from other causes. The survival pattern in most strains of rat also suggests that assay sensitivity is unlikely to be increased by extending a study beyond 2 years. As already pointed out, 2 years is approximately the 50% survival point, and the patterns of causation after this point are relatively constant. These patterns are dominated by three or four major entities, and the sudden appearance of a biologically and statistically significant increase in other lesions in the remaining rats could be difficult to detect. Similar considerations apply to many other strains of rat. The majority of morbidity and mortality is usually dominated by a small number of nonneoplastic and neoplastic conditions throughout the aging period.

The common morphological lesions can be grouped into degenerative, inflammatory, and proliferative lesions, the latter overlapping to some extent with neoplasms (Table 3.22). Most other lesions reported in aging control rats are of low incidence and of minor biological significance, and are not discussed. The biological significance of a lesion is equally as important as frequency, and the common lesions in Table 3.22 can be divided into two main groups on this basis. For example, degenerations of the kidney and nervous system can be lethal, whereas those in the liver and reproductive organs are usually incidental findings at necropsy. The main emphasis in this section will be on the clinically significant lesions and on lesions that cause problems in data analysis.

Table 3.22   Common Nonneoplastic Lesions in Aging Sprague Dawley Rats

Morphological Lesion	Clinical Condition
Degeneration	
Kidney	Glomerulonephropathy
Nerve	Radiculoneuropathy
Testis	Atrophy
Ovary	Atrophy/cyst
Liver	Steatosis
	Microcystic degeneration
	Telangiectasis
Inflammation	
Foot	Pododermatitis/arthritis
Tail	Dermatitis/folliculitis
Pancreas	Pancreatitis
Proliferation	
Liver	Biliary proliferation
	Altered cell foci/nodules
Adrenal	Altered cell foci/nodules
Mammary	Hyperplasia

Nonneoplastic Lesions

Nonneoplastic proliferations are a particular problem in analysis. The main use of long-term studies in rats is as a model bioassay for carcinogenicity. Indeed, there is a shift so that the rat may be the only species utilized for lifetime oncogenicity bioassays for many compounds. Nonneoplastic proliferations are seldom of clinical significance, but are extremely important because of the diagnostic difficulties they create in differentiation from preneoplastic lesions and from neoplasms. For example, spontaneous liver tumors are rare, but the liver is a common target organ of carcinogens, and, therefore, spontaneous proliferations are important because they mimic preneoplastic changes. However, the presence or absence of preneoplastic proliferative lesions associated with tumors in the same organ can be important in evaluating the carcinogenic potential of xenobiotics. In contrast, endocrine proliferations and tumors are common in rats, and the choice of criteria to differentiate nonneoplastic and neoplastic proliferations can profoundly alter the reported incidence of endocrine tumors in various laboratories. Johnson, in the initial section of this chapter, presents a current overview of tumor incidence rates found in rats in oncogenicity studies.

Kidney

Glomerulonephropathy: Of the degenerations, glomerulonephropathy is probably the major nonneoplastic condition in the aging rat of all strains (Gray, 1977), in that it accounts for a significant proportion of morbidity and mortality. The inbred Lewis rat appears to be less susceptible and the inbred Fischer 344 rat more susceptible to the development of chronic nephrosis than many random-bred rat strains (Bolton et al., 1976). Males are more susceptible than females. The severity of renal disease can be profoundly influenced by factors such as genotype and diet, and its presence hinders the evaluation of chronic nephrotoxicity.

Glomerulonephropathy has numerous synonyms, including nephropathy, nephrosis, and glomerulonephrosis; theories regarding its pathogenesis are equally numerous. The incidence and severity of disease are greater in males than in females. In advanced cases, the kidneys are enlarged, tan, and irregular in shape at necropsy, and the cut surface in severe cases may be grossly cystic. Histologically, both glomerular and tubular changes are present, but the latter are dominant. Some tubules are shrunken with thickened basement membranes accompanied by variable degrees of interstitial fibrosis. Other tubules contain hyaline casts or form large cysts filled with proteinaceous material. Glomerular changes are relatively inconspicuous and are characterized mainly by varying degrees of sclerosis and cystic dilation of Bowman's space. Such severe cases are unusual in 2-year-old rats, and in most instances, 50% or more of the kidney may appear reasonably normal.

The kidneys play a major role in fluid and electrolyte homeostasis and are important in the excretion of waste products. However, there is considerable reserve capacity, and a rat may survive for an extended period with severely damaged kidneys before dying of renal failure. Because the kidneys play a central role in fluid and electrolyte balance, advanced cases may lead to secondary patterns of pathology (syndromes) in other organs. The two main syndromes affect the cardiovascular system and calcium/phosphorus homeostasis.

Cardiovascular disease: The kidney receives 25% of the cardiac output and is intimately concerned with blood pressure regulation through the renin–angiotensin system. It is not surprising, therefore, that severe renal disease sometimes results in secondary cardiovascular disease. Myocardial fibrosis, especially in the left ventricle, is a common complication of severe renal disease, while the more advanced cases may show left atrial thrombosis or arteritis. Arteritis may be seen in one or more vessels, including renal vessels, but is not commonly recorded in the pancreatic, mesenteric, and testicular arteries. Advanced involvement of mesenteric vessels can be seen at necropsy as blue tortuous or nodular vessels in the mesentery around the duodenum and pancreas.

Histologically, the affected vessels show varying degrees of thrombosis, aneurysmal dilatation, fibrinoid necrosis, and leukocyte infiltration.

Mineralization syndrome: The mineralization syndrome is related to the kidney's role in calcium and phosphorus homeostasis via control of tubular resorption and also via synthesis of the 1,25-dihydroxy derivative of vitamin D3. The pathogenesis of the syndrome is incompletely understood, but renal impairment results in hyperphosphatemia and acidosis. Calcium phosphorus imbalance stimulates parathyroid hyperplasia, and there is increased bone resorption in an attempt to correct the hypocalcemia. Severe cases can be identified at necropsy. The parathyroids are grossly enlarged, the bones are thin and brittle, and the aorta is dilated and rigid owing to mineral deposited in the media. This mineralization affects many tissues histologically and is known as metastatic mineralization in contrast to the focal dystrophic mineralization (nephrocalcinosis) that is a common finding in the young rat.

Nerve

Radiculoneuropathy: Another major degenerative lesion in the aging rat is radiculoneuropathy. This disease may appear clinically as minor ataxia or as hind limb paralysis with urinary incontinence in severe cases. The lesion is predominant in males. Histologically, degeneration is seen in the posterior spinal cord (myelopathy), posterior nerve roots (radiculoneuropathy), and sciatic nerve (peripheral neuropathy) and results in secondary changes in the hind limb muscles (atrophy). Paralysis and urinary incontinence may predispose to infection of the urogenital tract, resulting in inflammation of the prostate (prostatitis), bladder (cystitis), or kidney (pyelonephritis). Inflammation in these organs can be severe and fatal in contrast to the minor lesions in young animals and illustrates the importance of qualifying terms in diagnoses.

Histological evidence of degeneration of myclin sheaths include vacuoles and voids within the sheaths, associated with foamy macrophages or, in severe cases, cholesterol deposits (clefts) and astrocytic, Schwann cell, or proliferation of fibroblasts.

Radiculoneuropathy is very common in aging males, but it is usually asymptomatic. In contrast, there is a less frequent degeneration of the central nervous system, which may produce obvious neurological symptoms, and the animal has to be removed from the study. This condition, termed encephalopathy, or more descriptively spongiform encephalopathy, consists of a multifocal vacuolation of the gray and white matter, most notable histologically in the cerebral cortex. Minor cases can be easily masked by artifacts, but the brain of old rats showing acute neurological symptoms in the absence of pituitary or brain tumors should be examined carefully for this condition.

Inflammations of biological significance are uncommon in aging rats unless they suffer from enzootic diseases such as mycoplasmosis. Prostatitis and other urogenital tract lesions are occasionally seen in animals with hind limb paralysis, but apart from these, lesions of the hind feet are the only common inflammations likely to make a significant impact on the health of the rat. Foot lesions are basically husbandry related and comprise two entities, pododermatitis and arthritis. Rats are commonly housed on grid or mesh floors, and males may reach 1 kg body weight on ad libitum feeding. It is not surprising, therefore, that this combination of high body weight and mesh floors occasionally results in lesions of the feet, particularly in the heavier males. Up to 5% of aging males may be removed from a study because of these foot lesions.

Foot

Pododermatitis. Pododermatitis is a local inflammation of the skin of the foot. It begins initially as a wartlike growth or callus on the foot pad due to constant pressure and subsequent proliferation of the skin. However, infection, ulceration, and bleeding may subsequently occur. Histologically, the lesion consists of inflamed granulation tissue variably covered by a hyperplastic squamous epithelium.

Arthritis. Arthritis is inflammation of the joint and surrounding tissues. Severe cases appear clinically as grossly swollen, firm, blue feet and hocks and may significantly impair the animal's mobility. The initial lesion is a periarthritis or tenosynovitis, resulting in marked periarticular edema with fibrosis and mononuclear leukocyte infiltration. The metatarsal joint ultimately becomes affected, and in some cases, the persistent chronic inflammation may incite a dramatic reactive bony proliferation that results in ankylosis of the involved joint.

Liver

The liver is such a frequent target organ in toxicity studies (in fact, the most common) that a discussion of some of the more common lesions that occur with aging in the rat seems warranted. More than half of aging male Fischer and Sprague Dawley rats have nonneoplastic hepatic lesions such as bile ductal hyperplasia and focal chronic hepatitis (Cohen et al., 1978). The National Toxicology Program has its own systemic nomenclature specifically for rat hepatoproliferative lesions (Maronpot et al., 1986).

Steatosis. Steatosis in the liver is a very general term, unless qualified further, and includes both focal and diffuse change, the latter being either centrilobular or periportal. The appearance of fat droplets is equally variable and may be either macrocytic or microcytic. The pathogenesis of steatosis may be equally diverse and includes dysfunction in the hepatocyte or imbalance in general lipid homeostatic. The latter is probably more common in aging rats because of obesity either due to ad libitum feeding or due to lipid mobilization in debilitated or clinically ill animals secondary to pituitary tumors or other debilitating lesions. In these cases, diffuse periportal steatosis is common.

Microcystic degeneration. Microcystic degeneration occurs in the liver and is also known as spongiosa hepatica. It may be visible at necropsy, but is usually a histological finding characterized by groups of thin-walled cysts containing pale-pink proteinaceous material and occasionally a few erythrocytes. The microcysts are thought to be derived from the fat-storing (Ito) cell in the liver. They are found mainly in males.

Telangiectasis. Telangiectasis is another common liver lesion. It is a vascular lesion, but is probably secondary to or associated with atrophy of the hepatic cords. It is most obvious at necropsy as depressed red foci on the surface of the liver and is particularly conspicuous when the liver is slightly yellow due to steatosis. Histologically, these foci are groups of dilated sinusoids usually located below the liver capsule. Telangiectasis is also very common in the adrenal cortex.

Biliary proliferations. Focal proliferations of the bile ducts are common in the liver. They are generally minor histological observations. Biliary proliferation (bile duct hyperplasia) consists of clusters or chains of bile duct–like formations lined by cuboidal or flattened epithelium. The proliferations are generally in the vicinity of the portal triad and may show varying degrees of basement membrane thickening or fibrosis. These structures do not progress to neoplasia, but the more basophilic cellular lesions could possibly mimic early-phase oval cell (ductular) hyperplasia, which is a response sometimes observed in the liver of rats fed carcinogens.

Foci of cellular alteration. Foci of cellular alteration, a controversial entity (from an interpretive viewpoint), is not uncommon in the liver of aging rats. It remains unclear whether foci of cellular alteration are preneoplastic or "progress" to adenomas. The focal proliferations consist of groups of hepatocytes that stand out from the normal liver parenchyma because of their arrangement, size, or tinctorial properties. They also demonstrate a variety of biochemical and functional properties such as the inability to store iron and increased r-glutamyl-transpeptidase activity, which can be utilized to distinguish foci of alteration from normal hepatocytes. However, not all foci of cellular alteration demonstrate consistent biochemical or functional properties in a given animal.

In routine H&E sections, foci are usually classified into vacuolated (clear), acidophilic, basophilic, or mixed cell foci. Clear X11 foci are characterized by "empty" cytoplasm representing the space occupied by glycogen or occasionally fat. The cells are usually larger than the surrounding hepatocytes. An increased amount of acidophilic cytoplasm is the characteristic feature of acidophilic foci, and the hepatocytes may also have an enlarged nucleus with a prominent nucleolus. In contrast, the hepatocytes forming basophilic foci are smaller than normal, and the cytoplasm contains prominent clumps of basophilic granules. Mixed foci, as the name suggests, contain hepatocytes of two or more of the previous types. Foci exceeding the size of a hepatic lobule are sometimes called areas of cellular alteration, and ones larger still that compress the surrounding parenchyma may be referred to as nodules. Basophilic foci are the most common and are encountered mainly in aging females, while most in males tend to be acidophilic foci. Overall, these altered cell foci and areas are uncommon in most strains of rat and do not significantly mask the interpretation of hepatocarcinogenic effects (Maronpot et al., 1986).

Hepatic cysts are, however, a common aging change in many rat strains. The lesion consists of cysts of different sizes that are lined by cuboidal or flat, endothelial-like cells. A moderate amount of connective tissue often surrounds the cysts. In the brown Norway rat, these lesions are especially common where large multilocular cysts often bulge from the hepatic surface (Squire and Levitt, 1975). Occasionally, large foci of endothelial-lined spaces containing eosinophilic amorphous material are found. The pathogenesis of these lesions is not clear, but they may represent a sequela to hepatic necrosis.

Adrenals

Altered cell foci. In contrast to the liver, altered cell foci areas and nodules are very common in the adrenal cortex, particularly in females. As in the liver, the lesions can be classified on the basis of staining characteristics into vacuolated, acidophilic, basophilic, etc., although there are subclasses of each type.

Finely vacuolated foci are found in both sexes and usually affect the zona glomerulosa and other zona fasciculata. Coarsely vacuolated foci are most common in males and usually lie in the central zona fasciculata. The small acidophilic cell focus (sometimes called hyperplastic focus) is often multiple and in the outer zona fasciculata. The lesion of most concern is the large acidophilic cell focus. These proliferations are common in aging females and may grow to grossly visible nodules of debatable diagnostic classification.

The proliferation of hyperbasophilic pheochromocytes in the adrenal medulla is an example of classification in the other direction. In this case, there is a reasonable continuity in both cytology and incidence to suggest progression from small basophilic foci to large metastasizing proliferations in rats nearing the end of their life span. Similar continuities are seen in the anterior pituitary and in the C-cell population of the thyroid. In all these cases, foci may be considered adenomas even though they are small, noncompressing, and with infrequent mitoses. There is no easy answer to these diagnostic problems, but they are often at the center of debate regarding the conclusions from carcinogenicity studies. It cannot be stressed too frequently that toxicologists and statisticians should appreciate the level of uncertainty that surrounds the classification of microscopic proliferations as hyperplasias or as neoplasias and evaluate the data accordingly.

Heart

Loss and degeneration of myocardial fibers with fibrosis is a common lesion of older rats. It occurs most frequently in the wall of the left ventricle or intraventricular septum and is characterized by areas of fibrosis surrounding myocardial fibers showing loss of striation, fragmentation, and vacuolization. The lesions vary from small foci involving only a few myocardial fibers to extensive

areas of fibrosis. The lesion becomes progressively more severe with age (Squire and Levitt, 1975). Cartilaginous foci at the base of the aortic valve are quite common in some Sprague Dawley strains and were once attributed to aging. However, similar cartilaginous foci have been observed in young rats (Hollander, 1968).

An unusual, but not uncommon, endomyocardial lesion has been described in several strains of rats. The lesion is characterized by proliferation of undifferentiated mesenchymal cells in the subendocardium and usually involves the left ventricle. The lesion is usually sharply demarcated from the myocardium but in places extends along muscle bundles or vessels. A few lymphocytes and occasional cell debris are found in the lesion. An occasional sarcoma is associated with endo-myocardial disease, but it is not known whether it represents a progression of the lesion. The lesion is definitely age associated. It is most common in rats over 30 months of age and is rarely observed in rats less than 27 months of age (Squire and Levitt, 1975). Myxomatous degenerative change of heart valves occurs in a majority of old rats.

Testes

Testicular atrophy may appear as a primary condition in aging rats, but it is also secondary to large pituitary tumors and to testicular arteritis. This is an example of a condition that may appear as a single entry on an incidence summary table, but its occurrence may be due to a variety of causes. Toxicologists and statisticians should be aware of the conditions in which pooling data due to different causes is commonly done. Atrophy of the seminiferous tubules ranges from focal uni-lateral to diffuse bilateral, and severe cases appear grossly as small, blue or brown, sometimes flac-cid, and watery gonads. Histologically, the tubules are shrunken and the seminiferous epithelium is lost, leaving only Sertoli cells. Interstitial edema is also frequent, and Leydig cell hyperplasia may occur.

Eye

Because the eye can be the target organ of toxicological studies, it is important to be aware of spontaneous lesions in order to avoid misinterpretation. In a study involving 400 rats up to 3 years of age, retinal lesions were found in more than one-third of the animals (Weisse et al., 1974). The lesions consisted of the loss of nuclei in the outer and inner nuclear layers of the retina, neuronal atrophy, and degeneration and thickening plus increased tortuosity of retinal capillaries. The lesions were both age and light dependent. The authors stated that while the lighting was not unlike that in other animal facilities, 12-hour light–dark exposures to less than 200 lux m/m^2 of light resulted in retinal lesions that appeared to be directly related to the amount of light exposure. There was retinal damage in 79% of the males and 96% of the females in the top rows of cage racks; 38% of the males and 50% of the females were affected in the bottom row. In a study involving Fischer 344 rats that were exposed to less than 10–320 lux m/m^2 of light, there was similar light-related retinal degeneration (Lai et al., 1978). In addition, the gradual loss of photoreceptor cells in rats that were exposed to less than 10 lux m/m^2 suggested to the authors that some cell loss is an age-related change. Peripheral retinal degeneration was unrelated to light intensity or severity of photoreceptor cell loss in other parts of the retina. Both studies indicate that age-related and light-associated retinal lesions are common and must be considered in long-term toxicological studies.

Skin and Appendages

The most common inflammatory lesion of the skin and appendages is inflammation of the hair follicle (folliculitis) in the tail. Nodules or postules are frequent along the tail and are primarily a

logistic problem related to GLP rather than a clinical problem. Theoretically, a tail nodule could be a tumor, and occasionally this is the case, but the vast majority are various stages of suppurative folliculitis. Many long-term study protocols state that all gross lesions will be examined histologically and a histopathologist will rapidly become an expert on rat tails (and ears or feet) if this requirement is adhered to strictly. These appendageal lesions could reasonably be treated in the same way as erect fur or fur loss in the skin, or as roundworms and tapeworms in the intestine. Mechanical abrasion from feeders is another possible cause. The clinical and necropsy data can be regarded as a definitive diagnosis in the vast majority of cases without the need to resort to histopathology.

Two other lesions of the integument pose similar problems. These are small nodules in the subcutis of the preputial region or on the back of males. These may be faithfully recorded week after week during the in-life phase of the study as palpable masses that grow slowly if at all, and some may regress. These two entities are preputial abscesses and squamous (epidermoid inclusion) cysts, respectively. Repeated palpation may aggravate and rupture the lesions and necessitate the removal of the animal from the study.

Pancreas

Another minor inflammatory lesion affects the pancreas. The histological appearance of this lesion is quite characteristic, but its status as a degenerative lesion (atrophy, microductular change) or postinflammatory lesion (adenitis, pancreatitis) is ill defined. There is focal loss of acinar epithelial cells, producing a ductular structure often accompanied by a mild interstitial inflammatory response. The lesion may be multifocal, but most of the exocrine pancreas is normal.

Neoplasia

Rats, like mice, develop a wide variety of tumors. Some of the factors known to affect tumor incidence include age at time of necropsy, strain of rat, sex, diet, and diligence with which tumors are sought. Careful macroscopic examination of the animal at necropsy plus multiple histological sections will result in a higher tumor incidence, especially of the smaller tumors. In this review, only some of the more common or controversial tumors are discussed.

Interpretation and Classification of Tumors

The two main criteria used in the classification of proliferations as tumors are morphology and the probability of progression. Morphological criteria include atypical cytology and organization. Nuclear cytoplasmic ratio, tinctorial properties, anaplasia, and mitotic rate are the main cytological characteristics that distinguish neoplastic cells from normal cells. Organizational atypia includes abnormal growth patterns, compression or invasion, and abnormal relationships between proliferating cells and blood vessels or other mesenchymal elements. These patterns serve to differentiate neoplasms from nonneoplastic proliferations.

Probability criteria (which incorporate both severity and incidence data) are mathematical assessments of the degree of association between small lesions of debatable classification and large clear-cut neoplasms. Crudely stated, if large, lethal masses are common in an organ, then common microscopic lesions are probably microscopic tumors or at least precursor lesions with a high probability of neoplastic transformation. On the other hand, if microscopic foci are common and gross tumors are rare in old animals, there is a low probability of any biologically significant degree of progression suggestive of neoplastic transformation. Pathologists use all of these criteria in assessing proliferations, but with different degrees of emphasis. This results in highly variable incidence

data for certain types of proliferative lesions. The reviewing toxicologist must become familiar with the main problem areas in a given study and evaluate the data in the perspective that application of diagnostic criteria may influence the incidence rates.

If the aforementioned criteria are applied to the large acidophil proliferations in the adrenal cortex, then on a morphological basis, large compressing nodules of cytologically distinct cells, sometimes with frequent mitoses, are common in the female adrenal. On this basis, there could be a 40% or more incidences of cortical tumors in some rat strains. Alternatively, the probable fate of many of these proliferations is to undergo vacuolar degeneration, resulting in a large blood-filled cyst that may thrombose. Further evidence against a neoplastic diagnosis is that large undoubted cortical neoplasms are often composed of small acidophilic cells cytologically distinct from the hypertrophied acidophilic cell of the commonly occurring nodules. Thus, if diagnosis is based on this cytological discontinuity and the high incidence of degeneration, these nodules would be regarded as hyperplastic rather than neoplastic.

The incidence and types of neoplasia are usually the patterns of pathology of most concern in long-term studies. The pattern of common tumors in the Fischer 344 was presented in detail in Table 3.23, and this is reasonably representative of other strains (except that more mammary tumors—up to a 55% incidence—are seen in Sprague Dawleys). The pattern is dominated by

Table 3.23   Reported Background Tumor Incidences in Fischer 344 Rats

Organ/Tissue	Chu (1977) M	Chu (1977) F	Fears et al. (1977); Page (1977); Gart et al. (1979) M	Fears et al. (1977); Page (1977); Gart et al. (1979) F	Goodman et al.[a] (1979) M	Goodman et al.[a] (1979) F	Chu et al. (1981) M	Chu et al. (1981) F	Chandra and Frith (1992) M	Chandra and Frith (1992) F
Brain	0.9	0.6	1.3	<0	8.1	0.55	.8	.06	0.3	0.1
Skin/subcutaneous	6.6	3.2	5.7	2.5	6.4	3	7.8	3.2	8.2	1.9
Mammary gland	1.4	17.9	0	18.8	1.54	8.5	1.5	20.9	0.5	14.6
Circulatory system	0.4	0.5	<1	<1	3.8	0.27	0.7	0.4	0.4	
Lung/trachea	3.1	1.8	2.4	<1	2.9	2	3.0	1.9		0.3
Heart	0.3	0.1	<1	<1	0.2	0.05				
Liver	1.8	3.1	1.2	1.3	1.74	3.9	2.2	1.9	0.9	0.3
Pancreas	0.2	—	<1	<0	0.16	0	0.2	—	6.5	0.1
Stomach	0.3	0.2	<1	<1	0.32	0.2	0.3	0.2		
Intestines	0.3	0.5	<1	<1	0.31	0.36	0.6	0.3		
Kidney	0.4	0.2	<1	<1	0.38	0.16	0.5	0.2	0.8	0.3
Urinary/bladder	0.1	0.2	<1	<1	0.1	0.22	0.1	0.3		0.1
Preputial gland	1.4	1.2	—	—	1.4	1.2	2.4	1.8		
Testis	80.6	NA	76.2	NA	80.1	NA	2.3	NA	79.5	
Ovary	NA	0.3	NA	<1	NA	0.33	NA	0.4		0.3
Uterus	NA	15.6	NA	16.8	NA	5.55	NA	17		14.1
Pituitary	11.5	30.5	10.2	29.5	11.4	0.3	4.7	34.9	70.5	30.3
Adrenal	10	4.6	8.7	4	9.95	4.58	2.4	5.2	7.3	1.2
Thyroid	7.1	6.5	5.1	5.6	7.16	6.65	8.2	6.8	7.6	6.3
Pancreatic islets	0.8	1	3.2	1.3	3.89	1.05	3.9	0.8	6.5	0.1
Body cavities	1.1	0.3	<1	<1	2.51	0.38	2.6	0.4		
Leukemia/lymphoma	11.7	9.1	6.5	5.4	12.3	9.9	9.9	13.4	30.5	20.5
N	1806	1765	846	840	1794	1754	[b]	[b]	740	740

[a] Gives detailed breakdown of neoplastic and nonneoplastic lesions in aged animals.
[b] Range of averages, six different laboratories.

subcutaneous connective tissue tumors, mammary proliferations, and by endocrine tumors. Other tumor types occur at incidence rates of less than 10% and in most cases rarely exceed 3%.

The rat represents the most commonly employed animal model in toxicological research and testing. As all other animal species, it has certain background incidences of lesions (particularly of neoplastic lesions), which are characteristic of it and which may confound interpretation of results. These vary from strain to strain, but there are general patterns for the species. Table 3.23 presents a summary of findings of tumor incidences in large groups of control Fischer 344 rats. These incidences show, in some cases, great degrees of variability. Haseman et al. (1989) discuss the major reasons behind such variability.

Pituitary

In several strains, one of the most prevalent spontaneous neoplastic alterations is the pituitary adenoma. Female Fischer 344 rats have been found to develop such tumors at a 30% rate by the time they were 110 weeks old in an investigation involving a total of 1754 females (Goodman et al., 1979), and most of the pituitary tumors were described as chromophobe adenomas. Carcinomas, on the other hand, consist of cells with anaplastic features and/or show invasion. Pituitary adenomas have also been observed frequently in other strains such as the Crl:CD(SD)BR strain (Cohen et al., 1978). Females appear to develop pituitary adenomas more often than males, although the incidence of such tumors in male F344 rats has been reported to be 14.7% (Coleman et al., 1977). In Wistar-derived SAG/Rij female rats, adenomas of the pituitary gland were found in 69% out of a total of 290 animals that had an average life span of 31 months (Boorman and Hollander, 1973). The incidences of spontaneous pituitary tumors in various rat strains have been reviewed by Carlton and Gries (1983).

Mammary Gland

Another frequently observed spontaneous neoplasm in the rat is the mammary tumor, the incidence of which varies from 10% to 40% depending on the strain and the age of the animals. While in WAG/Rij rats, pituitary tumors appeared to be the most usual cause of death in animals over 1 year of age, mammary tumors tended to develop later in life and were most often seen in animals that had survived for 2 years (Boorman and Hollander, 1973). Moreover, 68% of such rats with mammary tumors simultaneously revealed adenomas of the pituitary gland. Histologically, fibroadenomas and adenocarcinomas represent the most common varieties of benign and malignant mammary tumors.

Lymphoreticular System

Spontaneous tumors of the lymphoreticular system are fairly rare in rats, although a 25% incidence has been reported in Wistar Furth and Fischer strains (Moloney et al., 1969). In contrast to mice, such neoplasms have not been studied widely and are, therefore, not well classified. They usually progress with secondary involvement of the spleen, liver, lung, and often of renal adipose tissue. Generally, tumors of the lymphoid system are not common, but a 25% incidence has been reported in Wistar and Fischer rats (Squire and Goodman, 1978). Lymphoreticular cell tumors have not been widely studied and are not well classified. They usually involve the spleen with secondary involvement of liver, lung, and often the renal adipose tissue. Large granular lymphocyte leukemia (previously called mononuclear cell leukemia) is the most common "natural" cause of death in Fischer-344 rats used in chronic toxicity and carcinogenicity studies. Thirty percent to fifty percent of aging Fischer-344 rats in "control" populations die from the primary and secondary effects of this leukemia between 14 and 30 months of age (Losco and Ward, 1984).

Thyroid

After many strains have reached 2 years of age, neoplasms of the thyroid gland occur spontaneously at high frequencies. Again in WAG/Rij females, such tumors were found in 40% of 290 examined animals (Boorman and Hollander, 1973). In this study, electron microscopy revealed the cells of origin to be parafollicular.

Testes

Spontaneous testicular tumors are not common in the majority of rat strains (Sertoli cell tumors are especially rare in rats). However, a 100% incidence of interstitial (Leydig cell) tumors occurs in male Fischer rats by the time they are 30 months of age. Table 3.23 presents a summary of neoplastic lesion incidences in this strain (the most commonly used in long-term toxicology studies) as reported by various investigators.

Skin/Subcutaneous Tissues

Large subcutaneous masses are common finding in aging rats and may exceed 100 g in weight. In males, a variety of connective tissue tumors occurs, but they are usually fatty or fibrous, the latter predominating.

Fatty tumors (lipomas) are seen at necropsy as large, smooth, soft, glistening masses and aFe most easily defined in debilitated animals when the normal subcutaneous adipose tissue is depleted. In obese males, the distinction between small lipomas and large fat depots is not clear cut. Histologically, the lipoma consists of mature lipocytes sometimes with small bands of fibrosis.

Large Fibroma

Large, well-differentiated fibromas are the predominant subcutaneous connective tissue in males. Large tumors may impede movement or become ulcerated, and the animal has to be removed from the study. They appear grossly as well-circumscribed, firm, multinodular masses with a variable appearance on cut surface ranging from uniform white to a mosaic of white, cream-pink, and red areas. The histological appearance may vary widely within different areas of the same tumor. Fibroblasts are elongated cells producing collagen and ground substance, and the histological appearance of tumors depends on the arrangement of the cells and the relative proportion of cells and extracellular material. Most tumors contain abundant collagen, but in some areas, ground substance may predominate, producing a myxomatous appearance. The more cellular areas may suggest malignant transformation, but the fate of these cells appears to be differentiation rather than progression to fibrosarcoma, since cellular areas are common and metastases from these large subcutaneous masses are virtually nonexistent. Fibrosarcomas do occur, but are not common. Their characteristic histological feature is basophilia due to uniform hypercellularity in contrast to the largely eosinophilic fibroma. Mitotic figures are frequent and some tumors contain bizarre giant cells and multinucleate cells.

Dermal Fibroma

Another frequently encountered, but generally small mass, is the dermal fibroma. This is a distinct entity composed of an irregular mate of coarse collagen fibers similar to those of the normal dermis. Larger nodules extend into the subcutis. Both small and large nodules may contain sufficient adipose tissue to justify the combined diagnosis fibrolipoma. The relationship of this tumor to the large subcutaneous fibroma is uncertain, but it is more likely a separate entity rather than a precursor lesion.

Subcutaneous masses are more common in females than in males. They are frequently multiple and usually mammary in origin. The gross and histological appearance of these tumors is highly variable, but the majority are variants of a single entity, i.e., mammary fibroadenoma. The rat has six pairs of mammary glands consisting of milk-secreting epithelium and supporting or contractile stroma. Both epithelial and stromal elements proliferate, hence the diagnosis fibroadenoma. The degree of proliferation often varies in different parts of the same tumor, resulting in areas that are predominantly fibrous, predominantly epithelial, or mixed fibroepithelial. Diagnostic terms such as adenofibroma or fibroadenoma may be used to reflect the relative proportions of each component, but this division is probably unnecessary. Purely fibrous tumors may be impossible to distinguish from subcutaneous fibromas on one single section, but glandular formations may be found if multiple samples of the mass are examined. For statistical classification, it is reasonable to consider fibrous tumors in females as a variant of mammary fibroadenoma. Subcutaneous masses are the tumors most frequently seen as "palpable masses" in the in-life phase of long-term studies, but endocrine tumors are just as frequent in the final pathology phase. This group is dominated by the pituitary adenoma in both incidence and biological significance. Pituitary tumors are frequently visible at necropsy and are a common cause of morbidity and mortality. Thyroid C-cell tumors and adrenal pheochromocytomas are less common and frequently microscopic entities.

Endocrine System

Pituitary Adenoma

The anterior pituitary secretes several hormones and pituicytes fall into three main groups, acidophilic, basophilic, and chromophobe, depending on the tinctorial properties of the cytoplasm. In the past, attempts were made to use tinctorial classifications in the diagnosis of pituitary tumors, but this has largely been abandoned in favor of the nonspecific diagnosis pituitary adenoma or pituitary tumor. More sophisticated investigational techniques such as ultrastructure, immunocytochemistry, and hormone assay suggest that the majority of tumors secrete prolactin and the term prolactinoma is sometimes used.

However, in routine H&E sections, it is impossible to delineate functional properties, and it is inappropriate to use specific terms without any evidence for the functional status of the tumor. Pituitary adenomas are the most common tumors in several strains of laboratory rats (Boorman and Hollander, 1973; Cohen et al., 1978; Squire and Levitt, 1975). Pituitary tumors range in size from microscopic to macroscopic, raising the question of hyperplasia versus neoplasia. Since large lethal tumors 10 mm or more in diameter are common in this strain, the equally common microscopic lesions of similar cytology but just smaller in terms of size can reasonably be considered in the spectrum of adenomas. The microscopic appearance varies. In females, the cells are generally small to medium in size with relatively little cytoplasm (chromophobes). Dilated vascular channels are frequent and hemosiderin pigment is sometimes found. In males, the cytology is much more diverse, and bizarre pale eosinophilic cells are often seen. Microscopic tumors may be multicentric, and the larger tumors are nodular masses compressing the brain, often causing hydrocephalus. Most tumors are considered benign even though the cytology may be bizarre. Invasion of the meninges and along vascular channels into the brain is occasionally seen, but metastases are extremely rare.

The large tumors are space occupying and often functional and frequently result in other histological lesions. In females, acinar hyperplasia of the mammary gland and ovarian atrophy is commonly associated with pituitary neoplasms. Testicular atrophy may occur in males. Other components of the pituitary syndrome are splenic atrophy with hemosiderosis, squamous hyperplasia of the forestomach, and steatosis in the liver. The clinical syndrome of a thin, neurologically abnormal rat with red tear stains around the eyes is almost pathognomonic of large pituitary tumors.

Thyroid Tumors

Naturally occurring thyroid neoplasms occur with high frequency in many strains of rats, particularly after 2 years of age. These tumors have been shown to originate from C or parafollicular cells and to produce calcitonin (Boorman et al., 1972; Deftos et al., 1976; DeLellis et al., 1979). The lesions begin as diffuse or nodular hyperplasia of C cells. When the cells extend through the basement membrane or have distant metastases, they are called medullary thyroid carcinomas because they are similar to this tumor in humans. Unless one is aware of these tumors, they are easily missed because they are often microscopic. They tend to metastasize first to the deep cervical lymph nodes. Their malignant nature is more easily assessed if the lymph nodes are examined microscopically.

The thyroid C-cell proliferations are slightly more of a diagnostic problem than those in the pituitary. A minor degree of diffuse proliferation is common and is usually termed hyperplasia. Focal proliferations range from single perifollicular aggregates to grossly visible masses. Terminology varies, but proliferations occupying large areas of the thyroid are common, and the smaller foci could reasonably be considered part of the adenoma spectrum. However, other diagnoses used range from nodular hyperplasia to microscopic carcinoma depending on the diagnostic criteria applied by different pathologists. The cells usually form large pale acidophilic nests of round cells, compressing adjacent thyroid follicles. The larger tumors tend to incite fibrous encapsulation and occasionally show focal invasion of the capsule, surrounding tissues, or metastasis to the cervical nodes. These large masses are designated carcinomas, although the cytology and mitotic rate may not markedly differ from the smaller proliferations, hence the tendency for some pathologists to refer to all proliferations as carcinoma.

It has been reported that a variety of chemical carcinogens can also induce Zymbal gland tumors in the rat (Ward, 1975). Historically, finding tumors at this site in NCI studies performed in the early 1970s has led to inclusion of the tissue on standard necropsy collection lists.

Recent years have seen the availability of some wonderous comparative and descriptive resources for researching tissue specific pathological lesions (both neoplastic and otherwise) in the rat. Graves and Faccini, for example, provide an excellent descriptive text.

When evaluating tissues respective to prior or historical findings, however, one must be aware that baselines change over time. For example, the longevity of common strains (particularly the Sprague Dawley) has tended to decrease since the 1980s. At the same time, the shift in some quarters to restricted (as opposed to ad libitum) feeding in longer-term studies has changed control tumor incidence levels relative to past experiments.

DRUG METABOLISM

Christopher J. Kemper

This chapter will review what is known about the molecular biotransformation and transport mechanisms in the rat (*R. norvegicus*). While it is rare that nonclinical safety studies do not include the use of the rat as a model (Sprague Dawley or Wistar; Cayen, 2010), it is generally recognized that their similarity to humans is poor in many respects, from physiological parameters such as body weight and cardiac output to qualitative and quantitative differences in drug-metabolizing processes. This is reflective of the overall issue of whether animal species can predict the PK and toxicity in humans. While human cardiovascular, GI, and blood cell toxicities are predicted successfully from animal data in more than 80% of cases, overall, about a third of global human toxicities are not predicted in any of the standard species employed for drug safety assessment (Baillie and Rettie, 2011). But rodents are still used because of their easy availability and short life span. The latter allows for the growth of a large number of animals in a short period of time and, consequently, increasing the feasibility to conduct many pharmacological and toxicological studies (Martignoni et al., 2006).

Extrapolating study results from rats to humans is a challenge. In the past, many anatomic and physiological variables were correlated among mammals as exponential functions of body weight, using the power formula $Y = aW^b$, where Y is the physiological parameter, W is the body weight, a is the allometric coefficient, and b is the allometric exponent (Adolph, 1949). At the time, anatomic variables were (more or less) proportional to body weight, but the physiologic processes varied as the 0.7–0.8 power of body weight. In order to use a specific animal as a model for a physiologic or PK parameter, some sort of appropriate time scaling was required (Dedrick et al., 1970). The concept of physiological or PK time, in which a physiological or PK event becomes the independent variable, was explored by many scientists and was particularly embraced by Boxenbaum (1982). Unfortunately, a limiting factor in this type of PK scale-up appears to be the requirement of a very large database and, even then, the process failed more often than succeeded.

At about this time, a "game changing" factor in interspecies correlations appeared on the scene: the personal computer. Flexible mathematical tools also become available, such as fourth-order Runge–Kutta approximations for simultaneous ordinary differential equations. With software and hardware advances over time, physiologically based PK (PBPK) and PK and pharmacodynamic (PKPD) modeling became more and more realistic. A full discussion of PBPK/PKPD methods is beyond the scope of this chapter. Suffice it to say, this modeling is no longer an academic curiosity but an expected part of the drug development process. Case in point, the new draft FDA Guidance on Drug Interaction Studies (FDA, 2012) states:

> PBPK is a useful tool that can help sponsors better design drug-drug interaction studies, including trial and population pharmacokinetic studies, and quantitatively predict the magnitude of drug-drug interactions in various clinical situations.

Until recently, PBPK lacked many details about the systems they were trying to describe and hampered their use beyond simple allometric models. However, there has been a steady growth in biological pathway information and genetic data, information on molecular mechanisms, and technological advances in biological measurements, particularly with the universal availability of LCMS machines. These advances have made it possible to obtain the data needed for parameterization of more and more complete physiological models. With these models, it is easier (though still very difficult) to relate safety and efficacy results obtained from distinctly nonhuman test species to what would be expected in man. For details about PBPK processes, the reader is referred to the excellent book by Peters (2012). Table 3.24 is a list of some of the software tools that are commercially available to apply these processes.

Table 3.24   PBPK Software Tools

Berkeley Madonna	http://www.berkeleymadonna.com/	A simultaneous differential equation solver that requires some math skills
Cloe PK	http://www.cyprotex.com/insilico/	Includes PBPK modeling, in vitro ADME/PK and toxicology, and QSAR
MAXSIM2	http://www.maxsim2.com/	Moderately priced and very entertaining PBPK software with many preprogrammed physiological parameters
PKQuest	http://www.pkquest.com/	Free interactive PKPD program with preprogrammed human and rat data
Simcyp	http://www.simcyp.com/	Population-based modeling and simulation PKPD in virtual populations
Simulations-Plus	http://www.simulations-plus.com/	Library of widely used modules including GastroPlus (absorption modeling), ADMET Predictor (biotransformation estimates), MedChem Studio (in silico physiochemical properties)

General Considerations

Drug metabolism is a major clearance mechanism for three quarters of the top 200 prescribed drugs in the United States, primarily by Phase I and Phase II enzymes. P_{450}s and UGTs dominate those processes, and, in humans, CYP3A4 and UGT2B7 are the specific enzymes that, respectively, oxidize and conjugate the majority of drugs (Williams et al., 2004). In considering xenobiotic metabolism, one should also keep in mind the quantitative as well as qualitative differences between species. For example, at a low dosage (0.16 mg/kg) of benzo(a)pyrene, 1.7% will accumulate in the lungs of rats, whereas 7.9% accumulates in hamster lungs. At a high dosage, these percentages become 9.01% and 8.04% for rat and hamster, respectively (Weyland and Bevman, 1987). This is an example of a quantitative species-related difference.

At the low dose of benzo(a)pyrene, the predominant metabolites in all species were thioether (glutathione [GSH]) conjugates. In contrast, thioethers remained dominant at a high dose of benzo(a)pyrene in guinea pigs, but there was a considerable shift toward glucuronide formation in rats. This is an example of a more qualitative species-related difference. Studying metabolism in different species does not always mean that an investigator has to sort through a morass of species-related differences. Frequently, more similarities in metabolism exist between species than differences. For example, Berman et al. (1984) studied the *in vitro* microsomal metabolism of a-naphthoflavone (ANF) by various species. For the most part, all species produced ANF-5, 6 oxide, and ANF-dihydrodiol as the predominant metabolites. In addition, total hydrophobic (solvent extractable) metabolite formation was amazingly close between species, ranging from 2.7 to 3.2 nmol/15 min/mg microsomal protein. The reader is directed to the summaries on drug-metabolizing enzymes written by Lee et al. (2003) and Testa and Krämer (2010).

As in all species, the liver in the rat is the main site of xenobiotic metabolism. In general, the liver to body weight ratio in the rat is about 2.5%–3.2% in fasted rats and about 3.3%–4.0% in (ad libitum) fed rats owing to the differences in glycogen content. A sample of the characteristics of hepatic xenobiotic metabolism of the rat is summarized in Table 3.25.

Another consideration in studying species differences is the use of genotyping versus phenotyping (Testa and Krämer, 2010). Both are complimentary in information and need their own analytical tools. There are many reasons for species differences in drug metabolism despite the fact that most (if not all) enzymes have common ancestor genes. Differences are based on genetic variations leading to differences in enzyme expression levels, enzyme function and activity, different substrate specificities, different catalytic rates, and/or are present in liver at vastly different concentrations. Genetic and environmental factors are not always distinguishable when taking the phenotype approach. The advantage of phenotyping is that it provides direct information on the metabolite pattern and on enzyme function and activity. This chapter, therefore, will provide both genetic information and information on the phenotype. For example, none of the rat 2D enzymes or homologs (the genetic information) have the same substrate preferences as human CYP2D6 (the phenotype information). The term "homolog" refers to a related gene, which can be identified on the basis of sequence similarities, whereas "ortholog" is generally reserved for a related gene that maintains functional similarities.

Phase I Reactions

Cytochrome P_{450}: An MFO

General Background

Cytochrome CYP_{450}s (CYP_{450} enzymes, frequently abbreviated CYPs) form the single most important metabolic enzyme system in mammals, with many families and subtypes identified (Table 3.26). Although all members of this superfamily possess highly conserved regions of amino

Table 3.25   Summary of Major Hepatic Xenobiotic-Metabolizing Enzymes in Rats (–m, microsomal; –c, cytosolic)

Enzyme	Concentration or Activity	Comments and References
Cytochrome P$_{450}$ (microsomal)	0.20–1.00 nmol/mg-m 10–40 nmol/g (est.)	Variability due to differences in sex, age, and strain of rats used. When these variables are controlled, there is generally good agreement between animals (<10% difference). Astrom et al. (1986), Soulhaili-el Amri et al. (1986), Chengelis (1988c).
Cytochrome b5 (microsomal)	0.10–0.40 nmole/mg m 6–17 nmol/g (est.)	Differences with regard to strain, sex, and age are not as prominent as with cytochrome P$_{450}$. Astrom et al. (1986), Kiai et al. (1986), Souhaili-el Amri et al. (1986), Chengelis (1988c).
NADPH–cytochrome P$_{450}$ Reductase (microsomal)	75–200 nmol/min/mg m	For the more common strains. Fuller et al. (1972), Litterst et al. (1975), Soulhali-el Amri et al. (1986), Chengelis (1988c).

MFO activities
    Aminopyrene demethyl
    Aniline hydroxylase
    p-nitroanisole dimethyl
    Aryl hydrocar. hydroxyl.
	
	2.3–10.0 nmol/min/mg m
	0.9–1.3 nmol/min/mg m
	0.3–1.6 nmol/min/mg m
	0.3–1.25 nmol/min/mg m
		Determined under saturating conditions. Variations with age, sex, and strain. Page and Vessel (1969), Patil et al. (2008), Litterst et al. (1975), Astrom et al. (1986), Chengelis (1988), Koster et al. (1989).

EH
    (with styrene oxide)
    Microsomal
    Systolic
	
	2–12 nmol/min/mg m
	30–46 nmol/min/mg c
		Sex-, strain-, and age-related differences. Birnbaum and Baird (1979), Astrom et al. (1986), Chengelis (1988), Kizer et al. (1985), Oesch et al. (1988).

UGT
    1-Naphthol
    4-Nitrophenol
	
	5–40 nmol/min/mg m
	15–30 nmol/min/mg m
		Boutin et al. (1984), Galinsky et al. (1986), Astrom et al. (1987), Chengelis (1988).

GST
    CDNB
    4-Nitrobenzyl chloride
	
	360–1400 nmol/min/mg c
	110–380 nmol/min/mg c
		Gregus et al. (1985), Galisnky (1986), Chengelis (1988).

Table 3.26   CYP$_{450}$ Isozymes

CYP$_{450}$ Subfamily	Human Homolog	Rat Homolog
CYP1A	1A1, 1A2 (5%[a])	1a1, 1a2
CYP1B	1B1	1b1
CYP2A	2A6, 2A7, 2A13	2a1, 2a2, 2a3
CYP2B	2B6 (25%[a])	2b1, 2b2, 2b3, 2b12, 2b15, 2b21, 2b31
CYP2C[b]	2C8(1%[a]), 2C9(11%[a]), 2C18, 2C19(4%[a])	2c6, 2c7, 2c11, 2c12, various others
CYP2D	2D6 (30%[a])	2d1, 2d2, 2d3, 2d4, 2d5, 2d18
CYP2E	2E1 (4%[a])	2e1
CYP3A(52%[a])[b]	3A4, 3A5, 3A7, 3A43	3a1/23, 3a2, 3a9, 3a18, 3a62, 3a73
# Functional CYPs	~60	~90

Source: Baillie, T. A. and Rettie, A. E., *Drug Metab. Pharmacokinet.*, 26(1), 15–29, 2011. Epub 2010 Oct 22; Testa, B. and Krämer, S. D., *The Biochemistry of Drug Metabolism: Conjugations, Consequences of Metabolism, Influencing Factors*, Wiley-VCH, Zurich, Switzerland, 2010.

Note: Note the use of capital letters for the human genes and lowercase for the rat.

[a] Percentage of drugs affected in human PK.

[b] Additional members of the rat subfamily are listed in Table 3.25.

acid residues, there are relatively small differences in the primary amino acid sequences of the CYPs across species. However, even small changes in the amino acid sequences can give rise to profound differences in substrate specificity and catalytic activity (Martignoni, 2006).

There has been considerable variability in P_{450} nomenclature since the 1970s. The current accepted method is based on amino acid sequencing. Table 3.27 is only a partial list of corresponding names for the enzyme systems called Cytochrome P_{450}. For example, designations frequently utilized in this chapter include P-450a, b, c, or d. P-450c appears to correspond to a CYP1A isozyme and immunochemical analyses have indicated that P-450b belongs to the CYP3A subfamily. However, the identities of the P-450a and P-450d have remained unknown. More recent mass spectrometric techniques applied to characterize the proteins in historically archived samples of P-450a-d fractions revealed large heterogeneities of CYPs within the purified samples. Discussion herein using these older designations with impure materials was retained since the work described is illustrative of a particular property of the enzyme P_{450}, such as induction, and is not usually repeated with current methodologies.

Frequent use is made to what is refered to as the PubMed Gene database. PubMed is the name of a multifunctional, scientific database that is an essential tool for the researcher (see http://www.ncbi. nlm.nih.gov/pubmed). It has multiple gene components which I have collectively called the Gene database. An example is GenBank, which is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences. GenBank (http://www.ncbi.nlm.nih.gov/genbank/) is part of the International Nucleotide Sequence Database Collaboration, which comprises the DNA DataBank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL), and GenBank at NCBI. These three organizations exchange data on a daily basis. Much of the gene data in this chapter is derived from this source.

CYPs in rats were one of the first to be isolated and characterized (see Table 3.27). In fact, one of the oldest known CYP enzymes is rat form P450d, which has been shown to be similar to human counterparts. It is now named CYP1A2 enzyme, known to be conserved throughout the species with typical substrates as aromatic structures, preferably aromatic amines, but also polycyclic aromatic hydrocarbons and other planar structures. This form is inducible by polycyclic aromatic hydrocarbons as 3-methylcholanthrene (3-MC) or by polychlorinated biphenyls. A similar conclusion holds for the CYP2E1, which is in all species known to be inducible by ethanol and acetone and metabolizes organic solvents, nitrosamines, and several drugs, as, e.g., paracetamol (see earlier). The rat may serve as a readily available model for liver microsomal metabolism dependent on these two CYP forms.

Unfortunately, the rat is not a good model of metabolism for the most important human CYP family, namely, CYP3A, which affects over half of currently available drugs (Cayen, 2010). Many prototypical substrates of CYP3A enzymes as dihydropyridine calcium channel blockers (e.g., nifedipine) are not metabolized by rat CYP3A1 or by other rat CYP3A forms. The profile of CYP activities best resembling the human was seen in mouse followed by monkey, minipig, and dog liver microsomes, with rats displaying the most divergent. These data suggest that if hepatic xenobiotic-metabolizing characteristics were to be the sole reason for the selection of animal species for toxicity studies, then the rat might not be the most appropriate model to mimic human CYP activity patterns (Turpeinen et al., 2007). The most abundant CYP subfamily of rat liver is the CYP2C family, having a role similar to human CYP3A enzymes—which is supported by the fact that not only the oxidation of dihydropyridines as well as of the aflatoxin B1 but also hydroxylations of steroids are performed by rat 2C enzymes.

Another CYP enzyme important for drug metabolism, CYP2D6, quinidine, does not function well in the case of CYP2D1, but its stereoisomer quinine is a potent inhibitor in this case. Also, one of the marker substrates of CYP2D6, dextromethorphan, is metabolized specifically by another rat CYP2D enzyme, CYP2D2. There are probably significant differences in mechanism of induction of the CYP enzymes, as the rat CYP2D1 enzyme is inducible by 3-MC and phenobarbital (PB); however,

Table 3.27   Cytochrome CYP$_{450}$ Genetic Profiles

Gene Name	Family	Aliases and Other Designations	Summary
Cyp1a1	1	AHH; AHRR; CP11; CYP1; Cyp45c; P1–450; P450-C; CYP450DX; Cypc45c; P-450MC	Monooxygenase that plays a role in dioxin metabolism and detoxification, which is 3-MC-inducible
Cyp1a2	1	CYPD45; P-450d; RATCYPD45	Monooxygenase that plays a role in xenobiotic metabolism
Cyp1b1	1		Plays a role in polycyclic aromatic hydrocarbon metabolism in adrenal microsomes
Cyp2t1	2		Putative member of the cytochrome CYP$_{450}$ monooxygenase enzyme family
Cyp2a3	2	Cyp2a3a; RATCYP2A3A	Activates carcinogen N-nitrosomethylbenzylamine metabolism in the esophagus
Cyp2b2	2	Cype; CYP2B2	PB-inducible member of the CYP$_{450}$ xenobiotic-inducible superfamily that metabolizes nicotine and some drugs and activates carcinogens
Cyp2b6	2	Cyp2b6	Catalyzes reactions involved in drug metabolism and synthesis of cholesterol and steroids
Cyp2c11	2	Cyp2c; CYP2C2	Most abundant member of the CYP$_{450}$ xenobiotic-inducible superfamily in nonstimulated liver; metabolizes xenobiotics and testosterone
Cyp2c11l	2	Cyp2c11	Epoxygenase activity
Cyp2c22	2	Cyp2c70	Has similarity to class IIC subfamily cytochrome CYP$_{450}$ members including CYP4502C11 and CYP4502C12
Cyp2c23	2		Catalyzes the NADPH-dependent conversion of arachidonic acid to a mixture of epoxyeicosatrienoic acids in arachidonic acid metabolism
Cyp2c6v1	2	PB1; PTF2; Cyp2c6; CYP2C6	May play a role in drug metabolism
Cyp2d1	2	Cyp2d9	Debrisoquine 4-hydroxylase
Cyp2d2	2	Cyp2d26	Does not have debrisoquine hydroxylating activity
Cyp2d4	2	Cyp2d6; Cyp2d18; Cyp2d22; Cyp2d4v1; Cyp2d4v2	Has N-demethylation activity toward the antidepressant imipramine
Cyp2e1	2	Cyp2e	Facilitates hepatocyte sensitization to tumor necrosis factor-alpha–mediated cell death; may play a role in xenobiotic metabolism
Cyp2f4	2	Cyp2f1; Cyp2f2; CYP4502F4	Forms styrene glycol from styrene in liver and lung microsomes
Cyp2b15	2		Catalyzes reactions involved in drug metabolism and cholesterol biosynthesis
Cyp2b12	2	Cyp2b15	Catalyzes reactions involved in drug metabolism and cholesterol biosynthesis
Cyp2c13	2	Cyp2c38	Polymorphic cytochrome CYP$_{450}$ isozyme with male specific expression
Cyp2c6v1	2	PB1; PTF2; Cyp2c6; CYPIIC6	May play a role in drug metabolism
Cyp3a18	3		Catalyzes 16 beta- and 6-alpha hydroxylations of testosterone
Cyp3a2	3	Cyp3a11	Catalyzes the conversion of testosterone to 6-beta-hydroxytestosterone
Cyp3a23/3a1	3	CYP; RL33; cDEX; Cyp3a1; Cyp3a3; CYP3A23	Steroid-inducible member of CYP450 subfamily 3A; induced by pregnenolone 16 alpha-carbonitrile, DEX, PB, and triacetyloleandomycin

(*Continued*)

Table 3.27 (*Continued*)   Cytochrome CYP$_{450}$ Genetic Profiles

Gene Name	Family	Aliases and Other Designations	Summary
Cyp3a62	3	CYP3; Cyp3a	Has two isoforms
Cyp3a9	3	Cyp3a13; CYP450olf3	Catalyzes the hydroxylation of progesterone at 6beta, 16alpha and 21 positions
Cyp4a1	4	Cyp4a10; Cyp4a22; CYPIVA10	Arachidonic acid monooxygenase; catalyzes the hydroxylation of omega-terminal carbon of the arachidonic acid
Cyp4a2	4	Cyp4a11	Catalyzes the hydroxylation of arachidonic acid omega-terminal carbon to 20-hydroxyeicosatetraenoic acid
Cyp4a3	4	CYPIVA3; Cyp4a14	Liver monooxygenase; interacts with nitric oxide
Cyp4b1	4		May activate carcinogenic amines and contribute to bladder cancer
Cyp4f1	4	Cyp4f2; Cyp4f14	May be involved in hepatic carcinogenesis

Source: PubMed Gene database, http://www.ncbi.nlm.nih.gov/genbank/, accessed June 11, 2015.

Table 3.28   Rat Liver Microsomes

P450 Enzyme	Specific Content (pmol/mg Protein)	Percentage of Total Spectral P$_{450}$
Total P$_{450}$		
CYP4A[a]	300	3.0
CYP3A2	146	14.6
CYP2C[b]	650	65.0
CYP1A1, 1A2	12	1.2[c]
CYP2E1	79	7.9[c]
CYP2A1,2A2	54	5.4
CYP2B1,2B2	19	1.9

[a] Sum of CYP4A1 and 4A2.
[b] Sum of CYP2C6, 2C11, 2C12, 2C13.
[c] The rat is considered a good model system for these enzymes in man (Zuber, 2002).

the human CYP2D6 is known not to be inducible and 3-MC induces rather human CYP1A2 and PB is a classical inducer of human CYP3A, CYP2C, and CYP2B forms (Zuber et al., 2002).

Native concentrations of cytochrome P$_{450}$ range from 0.20 to 1.0 nmol/mg microsomal protein, depending on age, sex, and strain of rat examined. Table 3.28 shows concentration breakdowns for the major P$_{450}$ subfamilies. The rat was one of the first animals in which cytochrome P$_{450}$ was isolated and studied. And it was one of the first animals for which it was realized that cytochrome P$_{450}$ exists as a family of isozymes. As the literature in this field is extensive and complex, the reader is referred to reviews by Lu and West (1980), Nebert et al. (1981), Gonzales (1989), and Okey (1990), or the compilation assembled by Ortiz de Montellano (1986) for more details. The isozymic nature of cytochrome P$_{450}$ serves as a fundamental explanation for many of the characteristics of the rat mixed function oxidase (MFO). For example, aromatic hydrocarbon metabolism is high, induced by 3-MC because this agent induces an isozyme (once called cytochrome P-448 in the rat; now known as cytochrome P450c by most investigators) with high affinity for aromatic hydrocarbons. Also, the well-described differences in MFO between male and female rats are apparently due to different isozymes of cytochrome P$_{450}$ (Kamataki et al., 1985). Hence, we will continually refer to the isozymic nature of rat cytochrome P$_{450}$ in the ensuing discussions on age, sex, and hormonal effects on MFO.

The enzyme NADPH–cytochrome C reductase (also known as NADPH–cytochrome P$_{450}$ reductase) is the other main enzymic component of the MFO. It has also been isolated, purified, and well

characterized in the rat. Unlike cytochrome P_{450}, it does not exist as a family of isozymes. Only one enzyme exists and different isozymes are not induced by different agents. In fact, in the rat, PB and pregnenolone-16a-carbonitrile (PCN)-type inducing agents induce increases in both cytochrome P_{450} and the reductase, whereas 3-MC-type agents induce cytochrome P_{450}, but not the reductase in rats (Lu et al., 1972). The more effective inducer of the reductase than of cytochrome P_{450} is PCN, while PB is the better inducer of cytochrome P_{450}. There has been recurring debate as to whether the reductases of cytochrome P_{450} are the rate-limiting step in MFO activity, but it is generally believed in the activity of the reductase is the rate-limiting step in MFO activity (Mannering, 1971). Miwa et al. (1978) reported that incorporation of previously isolated reductase into freshly prepared microsomal preparations resulted in rate enhancements, which depended on the substrate examined and whether the microsomes were isolated from induced animals.

Across rodent species, the rat does not have either the highest concentrations of cytochrome P_{450} or levels of MFO activity. This was highlighted by Kato in his review in 1979. Amri et al. (1986) also noted that the male rat had less cytochrome P_{450} than either the guinea pig or the male rabbit. In addition, when maximal reaction rates were compared, the rat had the highest activities with only one substrate, benzo(a)pyrene. The guinea pig had the highest activities with four of the substrates (aminopyrine, benzphetamine, p-nitroanisole, and 7-ethoxycoumarin). This can have obvious toxicological consequences, such as with the higher sensitivity of rats as opposed to other rodents to chlorfenvinphos (an organophosphate insecticide) being due to a lower rate of metabolism in the rat. One should not assume, therefore, that the rat has the highest rates of metabolism for the chemical under study. In fact, there are several instances where a nonrodent, such as the dog (Duignan et al., 1987), has higher rates of oxidation for a substrate than the rat.

The previous paragraphs stressed quantitative differences between the rat MFO and those of other species. There are also qualitative differences. The rat has a high tendency to hydroxylate aromatic structures (e.g., benzene to phenol). The rat has a considerable ability to produce "phenolic" metabolites from chemicals like benzo(a)pyrene and aniline. Take the often-sited example of the metabolism of amphetamine. The major pathway in the rat is the formation of 4-hydroxyamphetamine, whereas in other species, the major pathway is through oxidative deamination to benzoic acid derivatives. There are exceptions. The rat, for example, lacks the ability to metabolize warfarin to 7-hydroxy-warfarin as other species do. Also, unlike many other species, the rat lacks the ability to oxidize the terminal nitrogen in aliphatic amines (Caldwell, 1981). N-Octylamine is, in fact, a very good inhibitor of cytochrome P_{450}–dependent MFO activity in the rat. Similarly, the rat produces relatively few deaminated metabolites in the metabolism of amphetamine.

Not all reactions catalyzed by the MFO (in the rat as well as other species) are oxidative in nature. Several reductive pathways of toxicological importance are also catalyzed by this system. For example, the carcinogenic activation of azo dyes by rat liver preparations has been extensively explored, with p-dimethylaminoazobenzene (DAB) being the prototypical agent. As reviewed by Zbaida et al. (1989), N-demethylation and N-oxidation reactions are involved in the activation of DAB to ultimate carcinogens, whereas detoxification is associated with C-oxidation and reductive cleavage of the azo bond. Their work clearly demonstrates the MFO is involved in the reductive as well as the oxidative reactions. The rat is obviously a good model for the study of these reactions. Interestingly, azo-reductase activity is highly inducible by β-naphthoflavone (β-NF) (a "3-MC-type" inducing agent that is commonly used because, unlike 3-MC, it is not carcinogenic). In the case of azo dyes, induction of "carcinogen-specific cytochrome P_{450}" leads to decreases in carcinogenic activation.

The MFO of the rat has been shown to have definite stereospecificity. Traiger and colleagues (Bush and Trager, 1985; Heimark and Trager, 1985) have published a very elegant series of studies on the metabolism of warfarin, demonstrating stereospecificity, in terms of both preferred substrate (R over S) and major metabolite formed (8-hydroxy vs. 6-hydroxy) in rats. Cook et al. (1982) examined the stereospecificity of disopyramide metabolism and observed in vivo that the (S)-enantiomer was more extensively metabolized. In addition, the major metabolite with the (S)-enantiomer was the

3-methoxyphenol metabolite, whereas none of this was produced from the (R)-enantiomer (which was largely excreted unchanged) where the predominant metabolite was mono-N-dealkylated disopyramide. Hence, in the rat, optical activity can govern both the rate and the pattern of metabolism. As one can judge on the basis of these two examples, it is difficult to determine a priori which enantiomer will be preferred and may vary with substrate. In addition, there may well be species differences; the preferred isomer in rats may not be preferred isomer in human subjects.

Inhibition (of the MFO)

The MFO of the rat is inhibited by a wide variety of chemicals. Discussion here is limited to the four different major classes of inhibition: (1) direct competitive agents, (2) suicide substrates, (3) synthesis inhibitors, and (4) antibodies.

Direct Competitive Agents

The classic competitive inhibitors of the MFO are represented by agents such as SKF 525-A, metyrapone, and 2,4-dichloro-6-phenyl-phenoxyethylamine (HCI), which avidly bind to the catalytic site on cytochrome P_{450}. SKF 525-A has been widely used by many investigators to block the metabolic activation of many indirect hepatotoxins. Generally, the dose administered is 75 mg/kg (intraperitoneally in saline) 30–60 min prior to the administration of the chemical under study. Murray (1987) reviewed these mechanisms of reversible inhibition associated with the imidazole or quinoline drugs. Ketoconazole, for example, is the prototypical imidazole antifungal agent that inhibits cytochrome P_{450}–mediated reactions in both the liver and the adrenal gland. In theory, any substrate for the MFO can competitively inhibit the metabolism of any other substrate, depending on the affinity constants, dosages, and isozymes of cytochrome P_{450} involved. Such interactions are, in fact, a major concern in polypharmacy, and the rat can be used for the study of these types of interactions.

Suicide Substrates

In contrast to the reversible inhibitors just discussed, the suicide substrates are irreversible substrates that are not necessarily distinctive on the basis of affinity or low turnover number. Upon oxidation by the MFO, they form reactive metabolites that irreversibly bind to and denature cytochrome P_{450}. Substrate specificity can be used to selectively inhibit specific cytochrome P_{450} isozymes. Chloramphenicol (300 mg/kg ip given in 0.5 mL propylene glycol) can lead to the destruction of only three of eight of the major cytochrome P_{450} isozymes as determined by their methodology. These included the major PB-inducible isozymes, whereas the isozymes induced by 8-NF, PCN, or clofibrate were unaffected by chloramphenicol treatment. Decker et al. (1989) have reported that 7a-thiosteroids specifically deactivate the rat cytochrome P_{450} induced by dexamethasone (DEX). Aryl alkynes inactivate 2B1, 2B4, 2B6, and 2B11 (Roberts et al., 1997).

Synthesis Inhibitors

Chemicals that inhibit cytochrome P_{450} synthesis or stimulate its breakdown will also have an inhibitory effect on MFO activity. Agents that generally block protein synthesis have been used to study mechanisms of induction, but lack specificity to be used as MFO inhibitors. Generally, agents that inhibit the synthesis of hemoprotein or stimulate hemoprotein deactivation (by increasing the activity of heme oxygenase) are more acceptable MFO inhibitors. Cobalt (as the chloride salt) inhibits heme syntheses and has long been used as a tool to inhibit the MFO-mediated activation of indirect hepatotoxins. The typical treatment regimen is 250 mmol/kg sc given 48, 36, 24, and 12 hours prior to hepatotoxic challenge (Chengelis, 1988a). As discussed by Spaethe and Jollow (1989), however,

the use of $CoCl_2$ as an MFO inhibitor has several disadvantages, in that its effect on cytochrome P_{450} is relatively brief and that other enzyme systems are affected as well. They discovered that cobaltic protoporphyrin IX can profoundly depress cytochrome P_{450} for 3–5 weeks. In rats, a single dose (90 mg/kg sc) led to approximately 90% depletion in cytochrome P_{450} 1 week posttreatment. This resulted in large decreases in the activity of the MFO toward all typical model substrates but had no effect on flavin-dependent mixed function oxidase (FMO), uridine-diphosphoglucuronic acid (UDP)-glucuronosyltransferase (UGT), 3'-phospho-adenosine-5'-phosphosulfate (PAPS)-sulfotransferase, or GSH S-transferase (GST) activities. Hence, Co-protoporphyrin may make a reasonable inhibitor to use as a tool for studying the involvement of the MFO in the metabolism and/or toxicity of a drug or chemical in the rat.

Antibodies

Antibodies to cytochrome P_{450} represent a fourth class of cytochrome P_{450} inhibitor. They have the disadvantage of being useful only for *in vitro* broken cell studies. The specificity of antibodies can be used to discern or confirm the specific isozyme of cytochrome P_{450} involved in a reaction. As discussed elsewhere, antibodies to NADPH–cytochrome c reductase can be used to discriminate between MFO- and FMO-catalyzed metabolic transformations.

There are a variety of miscellaneous other agents that inhibit the MFO by mechanisms that do not involve direct interaction with cytochrome P_{450}. These include agents such as menadione, which is a substrate for, and therefore competes with cytochrome P_{450} for, NADPH–cytochrome c reductase (Utley and Mehendale, 1989). In fact, it is the reduction of menadione by NADPH–cytochrome c reductase that is believed to be responsible for menadione cytotoxicity (Utley and Mehendale, 1989). Ethanol has also been repeatedly shown to inhibit MFO activity, but the mechanism is the subject of debate. There is little doubt, however, that at high concentrations (80–100 mM), ethanol inhibits the MFO. Both menadione and ethanol have pharmacological and toxicological properties that limit their usefulness as *in vivo* MFO inhibitors. For example, ethanol is not an MFO-potent inhibitor and, regardless of the underlying mechanism, would be a poor choice to use as an in vivo MFO inhibitor because of the CNS depression that would be caused at the dosages required.

Induction (of the MFO)

The fact that the activity of the MFO with different substrates responds differently to various inducing agents played a key role in the elucidation of the molecular biology of cytochrome P_{450}. Induction (or hepatic enzyme induction) refers to the process whereby treatment of an animal with a chemical results in an increased amount of endoplasmic reticulum, accompanied by an increased activity of the MFO. Induction will almost always result in an increased amount of microsomal protein, and this alone will cause increased MFO activity. In the strictest sense of the word, however, induction will also result in increased specific activity (on a milligram protein basis). The term induction had come to be applied loosely to both situations. In the rat, enzyme induction is almost always accompanied by increases in liver weight. In fact, the rat is a highly inducible species, and subchronic treatment with almost any organic chemical may well cause increases in liver weight. There are generally three classes of inducing agents, each class named for the prototypical agent: PB, 3-MC, and PCN. They are differentiable on the basis of the classes of cytochrome P_{450} isozymes they induce as well as the exact mechanisms of induction (as reviewed by Gonzales, 1989). Some agents are more specific than others. Mixtures of polychlorinated hydrocarbons (e.g., Aroclor 1254) induce both PB and 3-MC-type activities. It is for this reason that hepatic preparations used in the Ames Salmonella assay for mutagenicity are taken from rats treated with Arocolor 1254. Other enzymes could also be affected. PB, for example, will also induce increases in UDP-glucuronosyl and GSTs in the rat. With PB, good induction (approximately doubling cytochrome P_{450} with about a 30%

increase in liver weight) is obtained with three dosages of 80 mg/kg (intraperitoneally in saline with the pH corrected by HCl) over a 3-day period. For 3-MC-type induction, current practice is to use one dose of 100 mg/kg (intraperitoneally in corn oil) β-NF (which is not carcinogenic) followed by a 3-day rest period. Induction with PCN is usually affected by giving 25–50 mg/kg ip, for 3–4 days. Induction can provide a convenient tool for studying the metabolism of a specific chemical in the rat. For example, Decker et al. (1989) reported that spironolactone caused slight losses of cytochrome P_{450} in naive rats, but will cause much more profound losses in rats pretreated with DEX (a PCN-type agent that causes increases in cytochrome P-450p). This allowed for more thorough study of this phenomenon: the "suicide substrate" destruction of hepatic cytochrome P_{450} by spironolactone.

What are the implications of microsomal enzyme induction? The rat is a highly inducible species, and teleologically, this may explain why it tends to have lower baseline activity than other less inducible rodent species, such as the hamster. It is a rare chemical that (if given in a high enough dosage for a long enough period of time) will not have some inductive effect in the rat. Increases in liver weight with centrilobular hepatocyte hypertrophy (due to proliferation of the smooth endoplasmic reticulum) are a common finding in toxicity studies using the rat. This is a reversible, adaptive response and should not be considered (of and by itself) evidence of toxicity.

Alterations of intrinsic hormone metabolism can be another implication of induction. In the rat, a frequent example of this is increased metabolism and clearance of thyroid hormones (T3 and T4). While not cytochrome P_{450} mediated (rather these reactions are primarily catalyzed by UGT, discussed below in Table 3.36 and in the Glucuronide Conjugate section), they are still inducible. The increased clearance of thyroid hormones, a primary effect of microsomal enzyme induction, results in increased synthesis and release of the pituitary hormone TSH. Hence, an increased size and weight, and sometimes a frank increase in thyroid tumors in long-term studies, may be a secondary effect of hepatic microsomal enzyme induction.

Induction may be accompanied by other physiological changes that may have little to do with increases in xenobiotic metabolism. Induction of MFO in the rat often causes concomitant changes in plasma proteins. Typical clinical chemical panels in toxicity studies can result in the increased production of various microglobulins by the liver (Makarananda et al., 1987). Hence, MFO induction in the rat may be accompanied by slight increases in serum proteins associated with an increase in the globulin fraction. Such changes are of little, if any, toxicological importance. As discussed by Makaranaada (1987), however, chemical-induced liver damage will also cause changes in the components of the globulin fraction, albeit different ones than those changed by induction. One should not, therefore, jump to a conclusion concerning an increase in the globulin fraction without examining the liver histologically and/or examining plasma proteins by electrophoresis.

Another implication with regard to induction is that the rat is more inducible than most species. For example, Astrom et al. (1986) compared the effects of 3-MC induction in the rat, hamster, guinea pig, and two strains of mice. In rats, 3-MC produced a threefold increase in cytochrome P_{450} and a 100-fold increase in 7-ethoxyresorufin deethylation activity. In hamsters and guinea pigs, the same treatment induced only 1.5- to 2.5-fold increases in cytochrome P_{450} and five- to sevenfold increases in 7-ethoxyresorufin deethylation activity, respectively. In mice, the responses were strain dependent: 3-MC did not induce increases in DBA/2 mice, but did induce increases in cytochrome P_{450} and MFO activity in C57bl/6 mice. Hence, one cannot always extrapolate from the results obtained in rats to other species. In fact, it is likely that a weak inducer in rats will have little inductive effect in other species.

Another implication of induction in the rat is that increases in cytochrome P_{450} are not always accompanied by increases in MFO activity. The inducing agent may cause a change in the isozymic spectrum of cytochrome P_{450} such that the activity of the MFO toward some substrates may actually decrease. For example, Astrom et al. (1986) have reported that 3-MC induction resulted in decreases in the rate of aminopyrine metabolism *in vitro*. Rhodes and Houston (1983) have reported that PB caused a slight decrease in the formation of ^{14}C–CO_2 from ^{14}C-antipyrene *in vivo* in rats, whereas

P-NF caused a 50% increase. Ioannides et al. (1981) demonstrated 3-MC induction in rats enhanced subsequent benzo(a)pyrene mutagenicity in the Ames assay, whereas PB decreased it. The opposite occurred with 2-AAF induction. Thus, the type of induction (i.e., the isozyme of cytochrome P_{450}) can have important implications with regard to the toxicity of other agents in the rat. For example, as discussed by Lesca (1984), different mutagenic agents are preferentially activated by different cytochrome P_{450} isozymes: ethidium bromide is preferentially activated by 3-MC-inducible MFO activity. A single 80 mg/kg treatment of rats with 3-MC caused over a 100-fold increase in the number of revertant colonies in the Ames assay versus the response obtained with liver enzyme preparations obtained from control rats. PB and PCN, in contrast, caused only modest (2.5%–3.5%) increases. PB, however, did induce 100-fold increases in response in the Ames assay to cyclophosphamide, whereas 3-MC caused no increase in this response. Aroclor caused increases in the mutagenic activity of both ethidium bromide and cyclophosphamide, indicative of the broad spectrum of isozymes induced by this agent. Lesca (1984) recommended using changes in responses in the Ames assay to known mutagens to determine the nature of cytochrome P_{450} induced by new chemical entities in the rat.

Rats are a common species used for carcinogenicity testing. What are the potential implications of chronic exposure of rats to an inducing agent? Kurata et al. (1989) studied the long-term effects of PB on the liver and the MFO. The greatest degree of induction was achieved with 7 weeks of treatment. MFO activity and cytochrome P_{450} concentrations fell off slightly thereafter, but remained at a plateau 2.5- to 3.5-fold greater than control levels for the remainder of the study. Hence, induction has a finite effect: The liver cannot hypertrophy indefinitely. In pharmacological terms, rats accommodate to induction but do not become tachyphylactic. Many chemicals induce increases in their own metabolism. In a chronic toxicity study, plasma levels of the parent test article often decrease, whereas levels of metabolites increase during the test period. This can result in changes in toxicity depending on whether the parent or the metabolite is most responsible for toxicity.

Other than expected PK consequences does induction have any other implications? Lubet et al. (1989) explored the relationship between induction and hepatocellular tumor promotion. They suggested that potent inducing agents of cytochrome P-450b (e.g., PB, DDT) are all potent liver tumor promoters. Structural analogs (e.g., hexobarbital) that are not potent inducing agents are also poor liver tumor promoters. In addition, hamsters, where cytochrome P_{450} is not inducible, are resistant to hepatocellular tumor promotion. Hence, in chronic toxicity studies in the rat with inducing agents, promotion of naturally occurring "background" hepatic neoplasias is a real possibility such that a nongenotoxic chemical could appear to be a hepatic carcinogen.

Most of the implications of induction in the rat have to do with the specific isozymes of cytochrome P_{450} induced. Therefore, if in the testing of a chemical one has reason to believe that it is an inducing agent (gross increases in liver weight, centrilobular hypertrophy, increases in the grossly measurable cytochrome P_{450}), then identification of the specific isozyme of cytochrome P_{450} induced would aid in determining the implications of such findings. This can be accomplished without isolating and characterizing microsomal proteins by immunoelectrophoresis (Thomas et al., 1983, 1984) by simply looking at shifts in metabolic patterns in crude microsomal preparations. Wood et al. (1983) used changes in *in vitro* testosterone metabolism to discriminate or categorize types of induction. For example, a shift in *in vitro* metabolic spectrum that includes a large increase in 16p-testosterone formation would be indicative of cytochrome P-450a, or PB type induction in the rat. 7-Ethoxyresorufin is a specific substrate for 3-MC-inducible cytochrome P-450b, and Iwasaki et al. (1986) reported that 3-MC treatment (50 mg/kg ip for 3 days) increased 7-ethoxyresorufin deethylation 32-fold (from 0.05 to 1.6 nmol/min/nmol). These increases will appear even larger when corrected for increases in microsomal protein. Thus, the estimation of changes in the metabolic rate and/or metabolite profile for a handful of well-studied model MFO substrates can provide useful metabolic and toxicological information.

In summary, the phenomenon of microsomal enzyme induction has been well studied in the rat. Different chemical classes have somewhat different specific effects, but, in general, most of the

implications of microsomal induction are due to the increases in specific isozymes of cytochrome P_{450}. The rat is a highly inducible species, but there are age-, sex-, and strain-related quantitative differences. Given that, and also given the implications induction may have for the metabolism and toxicity of a chemical in the rat, the careful investigator should consider inspection of the type of induction related to a specific chemical under study. The technology for doing so is available and easily adaptable.

Microsomal induction studies generally involve biochemical analyses conducted at a single time point following treatment with the suspect inducing agent. One needs to be cautious in the design and interpretation of such studies, however, as different isozymes have time courses of response to different inducing agents. For example, Parkinson et al. (1983a) examined the time course of the response of Long-Evans rats to a single dose of Aroclor 1254 (500 mg/kg ip). Cytochrome P-450c was maximally induced 2 days after treatment and remained constant. In contrast, cytochrome P-450a was not maximally induced until 9 days after treatment. Other isozymes had yet different patterns. All isozymes started to decline 15 days following treatment. Hence, if one is concerned about the nature of the hepatic induction affected by a poorly characterized chemical in the rat, several time points should be examined.

Sex-Related Differences

The hepatic MFO is under a variety of hormonal controls that are responsible for the well-reported differences in male and female rats. In fact, sex-related differences have been most frequently and convincingly demonstrated in the rat as opposed to other species. Hormonal control of drug oxidation is very complex but has been most well characterized in the rat. This effort dates back to the late 1950s and early 1960s, when it was first noticed that there was a sex difference in drug metabolism, with males generally having higher activity than females. For example, Kato et al. (1964) demonstrated that male Wistar rats had consistently higher in vitro rates of microsomal aniline, aminopyrine, and strychnine metabolism. Furner et al. (1969) reported that regardless of the strain of rats examined, male rats generally had higher rates of microsomal metabolism with aniline, aminopyrine, ethylmorphine, and other model substrates. The extent of this difference varied depending on age, strain, and substrate examined. These earlier works were among the first to also note that the sex-related difference was less apparent in sexually immature rats. This has also been confirmed repeatedly and will be discussed in greater detail below (see Age-Related section on p. 239).

Ariyoshi et al. (1981) demonstrated in Wistar rats that there was no difference in cytochrome P_{450} content between males and females at 30 days of age, but there were large differences at 300 days. The fact that the sex-related differences in xenobiotic metabolism were less apparent in sexually immature rats led to many experiments exploring the hormonal control of MFO activity. The work in this area has been reviewed in detail by Skett (1988).

In general, there are substantial data suggesting that the androgenic and estrogenic hormones exert different and competing modulating effects on MFO activity. For example, early work demonstrated that castration reduced activity in males to female levels and testosterone supplementation "remasculinized" drug metabolism in rats. The differences are due to differences in the total amount of as well as the spectrum of cytochrome P_{450} isozymes induced. Kato and coworkers (Kamataki et al., 1985) have identified two distinct (by immunological methods and by electrophoretic mobility) forms of cytochrome P_{450} (termed cytochrome P_{450}-male and cytochrome P_{450}-female) that are specific to the respective sexes in Fischer 344 rats. Cytochrome P_{450}-male is associated with a higher rate of MFO activity with several model substrates (e.g., 7-propoxycoumarin, aniline, and benzphetamine) than cytochrome P_{450}-female. High levels of cytochrome P_{450}-male are induced by testosterone: castration leads to decreases in this isozyme.

In contrast, cytochrome P_{450}-female is present only in female rats, and the levels are attenuated after oophorectomy and exogenous testosterone administration. Other hormonal effects on MFO activity have been reported. Growth hormone (a pituitary hormone) has been shown to have a

feminizing effect on xenobiotic metabolism in the rat (Skett, 1988), also presumably due to differences in expression of cytochrome P_{450} isozymes. Waxman et al. (1985), for example, have described a distinct isozyme of cytochrome P_{450} (termed P4502c(male)/UT-A), which is responsible for testosterone 16a-hydroxylation activity in liver microsomes from male rats. Castration of male rats at birth led to diminished amounts (to levels found in female rats) of both this cytochrome P_{450} isozyme and 16a-hydroxylation activity.

While hormonal effects on MFO activity are generally attributed to control and expression of specific cytochrome P_{450} isozymes, other actions are also involved. Waxman et al. (1989) demonstrated that hypothesectomy resulted in elevated amounts of several cytochrome P_{450} isozymes and decreases in others, but there was little correlation between the changes in cytochrome P_{450} with changes in MFO activity. They demonstrated that this was due to decreases (26%–30% of control) in NADPH–cytochrome P_{450} reductase activity and was restored by exogenous T4 treatment, but not by other hormones. They concluded that the reductase is subject to hormonal controls that are distinct from those for cytochrome P_{450}. This observation is also consistent with the observation that reductase activity is also the rate-limiting step in MFO reactions.

In summary, the well-described sex-related differences in MFO activity (toward xenobiotics and endogenous steroid hormones) in the rat (with males generally having higher activity than females) are due to the implicit hormonal differences that result in the expression of different isozymes of cytochrome P_{450} and total NADPH–reductase activity. Hence, it is not at all uncommon for a chemical to have different PK behavior in male versus female rats, or for there to be quantitative differences in chemical toxicity between the two sexes of this species. In addition, as the use of surgically altered rats in pharmacological experiments is not uncommon, these potential effects that the resulting hormone changes may have on drug metabolism should be kept in mind.

Strain-Related Differences

Strain-related differences in MFO activity and inducibility have long been recognized, as Conney (1967) mentioned in an early review on the subject of microsomal drug metabolism. In their classic 1969 article, Page and Vesell compared native and induced rates of *in vitro* (with S-9 fractions) oxidation of ethylmorphine and aniline in 10 different strains of rat. They noted several strain-related differences. For example, baseline aniline hydroxylation activity in males varied from 6.9 (Wistar) to 20.2 nmol/min/mg (Long-Evans). Interestingly, there were these baseline differences in activity despite the fact that cytochrome P_{450} content did not differ substantially between strains. Following induction by PB, activity tripled in Wistar rats but increased only 50% in Long-Evans rats. More recently, Koster et al. (1989) examined strain-related differences in the MFO of 14 highly inbred strains that had originated from four different European facilities and identified considerable strain-related variations. Kai et al. (1988) determined that Sprague Dawley rats had lower amounts of cytochrome P_{450} as well as lower MFO activity with the model substrates aniline and aminopyrine than Wistar rats. The Sprague Dawley rat, however, was more inducible with malotilate than the Wistar rat. Augustine and Zemaitis (1989) also demonstrated that there were significant differences in MFO activity between Wistar, Sprague Dawley, and Fischer 344 rats. Jackson and Li (1987) reported that citral was a more potent inducer in Long-Evans than in Wistar rats.

There are also strain-related differences in the metabolism of endogenous substrates. Shefer (1972), for example, compared testosterone 7a-hydroxylation activity in Charles River Sprague Dawley (CD-1) and Wistar rats. Activity was 3.59 and 2.36 nmol/min/mg (approximately 50% difference), respectively. Following PB treatment, there was essentially no change in this enzyme activity in Sprague Dawley rats, but a sixfold increase in Wistar rats. While there are exceptions, three generalizations tend to emerge from the literature with regard to strain-related differences in MFO activity in rats: (1) In all strains, males have higher activity than females. Quantitatively, this difference is greater in the Fischer 344 and Long-Evans strains than the Sprague Dawley and Wistar

strains. (2) Baseline (native) activities in Wistar rats tend to be lower than those of the Fischer 344 and Long-Evans strains, and the Sprague Dawley strain tends to be intermediate. (3) The Wistar strain tends to show the greater inductive response to PB-type inducers.

Strain-related differences are an important consideration in the design and interpretation of toxicity studies, but they can also be used as specific experimental tools. The Lewis and DA rats are closely related strains that differ in that the DA rat has poor hepatic debrisoquine 4-hydroxylation activity (Kahn et al., 1985). Tucker et al. (1980) made interesting use of strain-related differences in rats to study the metabolism and toxicity of carbon disulfide. In their hands, the extent of CS_2-mediated hepatic hydropic degeneration varied considerably in four different inbred strains of rats and was accompanied by hepatic necrosis in the most susceptible strain. There was also a good correlation between the extent of hepatic damage and the CS_2-mediated loss of cytochrome P_{450} (CS_2 being a suicide substrate). Hence, while the exact mechanism is unclear, these results demonstrate that the loss of cytochrome P_{450} is involved in the mechanism of CS_2-mediated hepatotoxicity. These examples demonstrate the utility of using strain-related differences for studying the relationship between metabolism and toxicity of a drug or chemical.

Celier and Cresteil (1989) described an interesting strain-related phenomenon involving the Gunn rat. As discussed elsewhere (see under conjugation reactions), this strain has a well-characterized lack of UGT activity. Celier and colleagues further demonstrated that the Gunn rat also does not respond to 3-MC induction with increases in total cytochrome P_{450}, but does develop increases in MFO activity with substrates such as 7-ethoxyresorufin, generally associated with this type of induction. Immunoquantification methods demonstrated that 3-MC-treated male (a different pattern was seen in females) Gunn rats had increases in cytochrome P-450c and P-450d, but with an equivalent decrease in other isozymes. A more usual inductive pattern would have been an increase in specific isozymes, while the levels of others remained constant. Interestingly, while 3-MC has been shown to induce UGT (as well as cytochrome P_{450}) in other rat strains, it does not induce increases in Gunn rats. The example of the Gunn rat provides an interesting example of a sex difference nested within a strain difference in inductive response. This highlights the need to be very cautious in interpreting the implication of microsomal induction in the rat.

The Zucker rat is a strain with a marked tendency to develop obesity and is frequently used as a model to study the physiological consequences of this condition. This strain is not commonly used to study toxicological problems, but does provide an interesting example of strain-related differences that can be tied to phenotypic expression. Brouwer et al. (1984) compared the inductive response of lean versus obese Zucker rats to Sprague Dawley rats and found that PB treatment caused similar increases in Sprague Dawley and lean Zucker rats, that is, increases in antipyrine clearance, liver weight, microsomal protein, and cytochrome P_{450} content. The obese Zucker rat, however, failed to respond to PB treatment with increases in any of these parameters.

Another implication in considering strain-related differences is illustrated by the case of PCN. There has been some debate on whether or not PCN induced increases in benzo(a)pyrene metabolism in the rat. This disagreement resulted from strain-, age-, and sex-related differences, as reported by Gorski et al. (1985). They demonstrated that PCN did induce increases in benzo(a)pyrene metabolism in 1-month-old rats regardless of strain (Long-Evans, Sprague Dawley, Wistar, or Holtzman). Differences began to develop as the rats matured. In male (but not female) Long-Evans rats, for example, inducibility of benzo(a)pyrene metabolism with PCN decreased with age. Hence, the age, sex, and strain of rat used as well as the model substrate and assay techniques can all influence the apparent inductive response in the rat. It is not unusual to see this type of disagreement in the literature. This example also underscores the need to pay close attention to experimental details, such as the sex, strain, and age of rats used when one needs to replicate the work of another investigator.

While strain-related differences in metabolism may often be the basis for the differences in toxicity, this is not always the case. For example, acetaminophen is nephrotoxic in Fischer 344 rats but not in Sprague Dawley rats. Tarloff et al. (1989) extensively explored the metabolism and PK

of acetaminophen in age- and sex-matched rats for these two strains and could not identify any differences to account for the strain-related differences in toxicity. Further, Newton et al. (1983) were unable to identify any specific differences in renal xenobiotic metabolism that could provide an explanation for the difference in acetaminophen nephrotoxicity in these two strains of rat. Elsewhere, Plummer et al. (1987) were unable to identify any metabolic differences between Fisher 344 and DA rats that could account for the strain-related differences in sensitivity to aflatoxin. In other studies (Kahn et al., 1987), this group was also unable to demonstrate differences between DA and Fischer 344 rats with regard to phenacetin metabolism.

Age-Related

Age-related changes in xenobiotic metabolism, particularly that affected by the MFO, have been studied in the rat for quite some time. In general, these studies fall into two categories: those on postnatal or perinatal changes and those on senescent changes. Perinatal changes will be discussed first.

In 1964, Kato et al. claimed (quoting earlier works by Fouts and Jondorf) that "it is a well known fact that in new born animals there is a deficiency in certain drug metabolizing enzymes." They demonstrated (using female Sprague Dawley rats) that the *in vitro* microsomal metabolism of five different model substrates was quite low in neonatal rats (approximately 5% of the activities observed in 30-day-old rats), but these activities increased gradually and steadily to a peak in 30-day-old rats and declined thereafter. They also demonstrated that there were age-related changes in NADPH–cytochrome P_{450} reductase that paralleled the changes in MFO activity.

Nearly 10 years after the publication by Kato et al. (1964), Muller et al. (1973) published on age-related changes in cytochrome P_{450} in male Wistar rats; the concentrations in 10-day-old rats were less than half of those observed in adult (30 to 60 days old) rats. Barbital was a poor inducer in young rats. This paper also noted that changes in MFO with age did not correlate well with the amount of cytochrome P_{450}, but did not mention the earlier work by Kato. The additional literature in this area through 1980 is summarized by Klinger (1982). While he questioned whether the activity of NADPH–cytochrome P_{450} reductase is the rate-limiting step in all MFO activities, his review clearly indicates that neonatal rats have low MFO activity, which may be due to a combination of factors: low amounts of cytochrome P_{450} (not always seen by all investigators), the low activity of NADPH–cytochrome P_{450} reductase, and the isozymic character of the cytochrome P_{450} present.

Depending on the model substrate, there are different time courses of MFO development and responsiveness to inducing agents. Devasagayam et al. (1983), for example, reported that female Wistar rats at 1 day of age have 25% of the cytochrome P_{450} and 20% of the NADPH–cytochrome P_{450} reductase as those at 75 days of age. While there are exceptions, in general, the sex-dependent differences in MFO activity are not apparent in neonatal rats and tend to develop concurrently with sexual maturity. Waxman and colleagues (1985) demonstrated that this was because different gonadal hormones control the levels of different cytochrome P_{450} isozymes. For example, both males and females are born with very low testosterone 16a-hydroxylating ability. This increases rapidly in males, but remains low in females even after sexual maturation (4–6 weeks of age).

The quantitative and qualitative responsiveness of neonatal and perinatal rats to inducing agents is still a matter of some debate. In contrast to the aforementioned results with barbital, Pyykko (1983) reported that perinatal (3 days old) rats are as responsive to the inducing effects of toluene as adults. In toxicological research, experiments that require the treatment of neonatal rats are probably quite rare. The inducibility of 2-day-old rats may be of intellectual interest, but the implications in developmental/reproductive toxicity are not clear. In contrast to inducibility, the fact that neonatal rats have a low metabolic capacity has definite toxicological implications. Depending on the toxicological mechanism of action of a chemical, neonatal rats will respond differently than adult rats.

The activity of MFO in rats has also been shown to change with advancing age. Kato et al. (1964) also discussed changes in senescence. In general, MFO activity appears to decrease as rats age. For example, Kato (1964) noted that cardosopil metabolism peaked at 30 days of age and declined progressively thereafter. The rates of plasma clearance were equivalent in 15- versus 250-day-old rats. Baird et al. (1975) reported that there were age-related (30 to 900 days old) changes in CFN male rats, in that while the concentrations of cytochrome P_{450} did not decline appreciably, *in vitro* microsomal metabolism of zoxazolamine did, and this decline seemed to reflect an age-related decline in NADPH–cytochrome P_{450} activity. Rikens and Notley (1981, 1982a,b) examined age-related changes in male Fischer 344 rats and found that microsomal cytochrome P_{450}, cytochrome b5, and NADPH–cytochrome c reductase decreased in middle-aged (14–15 months) and old (24–25 months) rats compared to young rats (3–5 months). Changes in MFO activity were variable (sometimes increasing, sometimes decreasing with age) depending on the substrate. Schmucker and Wang (1980a) reported the levels of cytochrome P_{450} and NADPH–cytochrome c reductase were highest in middle-aged (16 months) rats, with marked decreases in older rats (27 months). The MFO activities tended to be lowest in older rats (Schmucker and Wang, 1980b). Additionally, they reported that the microsomal cholesterol/phospholipid ratio increased in senescent rats. The resulting change in membrane fluidity was thought to change the interaction between the reductase and cytochrome P_{450} (Schmucker et al., 1984).

While age-related changes in MFO activity in Fischer 344 rats have been extensively studied, relatively few reports on age-related changes in Sprague Dawley rats have been published. Chengelis (1988a) reported a rather extensive investigation of age-related changes in MFO activity in male and female Sprague Dawley rats. In a pattern similar to that observed in Fischer 344 rats, cytochrome P_{450} peaked (on a gram liver basis) in rats at 39 weeks of age. NADPH–cytochrome P_{450} reductase activity also peaked at 39 months of age and declined thereafter. In senescent rats (greater than 7–8 weeks of age), there were apparent differences between sexes with regard to the levels of the components of the MFO and MFO activity. Interestingly, there was little correlation between MFO activity and the amounts of cytochrome P_{450}. For example, the activity toward p-nitroanisole was lowest in 39-week-old rats (when cytochrome P_{450} was highest). Aniline hydroxylase was lowest in 104-week-old rats. These results suggest that there are not only age-related changes in total cytochrome P_{450} but also age-related changes in the isozymic spectrum. This would be consistent with the earlier findings by Kamataki et al. (1985), who reported that there were definite age-related changes in male Fischer 344 rats. Benzo(a)pyrene hydroxylation and 7-propoxycoumarin dealkylation activities peaked at 6 months of age in males, but not in females where activities remained constant throughout adult life. In males, there was a drop in MFO activities after 24 months of age to female levels. For benzo(a)pyrene hydroxylation, for example, peak *in vitro* activity was approximately 350 pmol/min/mg microsomal protein in 6-month-old male rats and approximately 90 pmol/min/mg in both sexes at 24 months of age. These changes directly reflected the amounts of cytochrome P_{450}-male as well as the appearance of cytochrome P_{450}-female in senescent male rats. Hence, while there are quantitative and qualitative exceptions, depending on strain, sex, and age of the rats as well as the substrate of interest, MFO activity in rats tends to be highest in middle-aged rats (9–14 months) and lowest in senescent rats (24 months). Age-related changes are due to a combination of changes in total cytochrome P_{450}, the spectrum of cytochrome P_{450} isozymes, NADPH–cytochrome P_{450} reductase, and microsomal lipid content. A further complication is that microsomal protein will also change with age (Chengelis, 1988c). Because of this variability, one should not base too many conclusions on these generalizations without specific examination.

Given there are age-related changes in cytochrome P_{450} in rats, can there also be age-related changes in response to inducing agents? Age-related changes in the sensitivity of rats to inducing agents have been discussed since the late 1960s. The literature in this field is mixed. For example, Kato and Takanaka (1968a,b) reported that PB (60 mg/kg ip for 3 days) caused much larger increases in 40-day-old than 300-day-old rats: 23% versus 8% in microsomal protein, 249% versus

52% in NADPH–cytochrome c reductase, 259% versus 60% in cytochrome P_{450}, and 713% versus 173% in aminopyrine N-demethylation activity. Kao and Hudson (1980) compared and contrasted the inducing effect of PB (80 mg/kg ip for 4 days) versus β-NF (80 mg/kg ip for 4 days) in young (10 weeks) versus old (100 weeks) male Fischer 344 rats. As expected, the baseline amount of MFO components as well as the MFO activities examined was higher in young-adult rats than senescent rats, but both agents caused comparable degrees of increase in both age groups. They concluded that young and old rats responded to inducing agents in relatively the same quantitative fashion. In contrast, Rikans and Notley (1982a,b) reported that age affected the response to different inducing agents differently. They concluded that PB induced increases to approximately the same extent in old versus young rats, whereas β-NF induced larger increases in older rats. They further suggested that age-related differences in sensitivity to induction are due to differences in the inducibility of specific cytochrome P_{450} isozymes. This latter conclusion is consistent with the work of Sun et al. (1986), who examined the levels of six different forms of cytochrome P_{450} as a function of both age and inducing agents using immunochemical techniques. They noted that PB induced a different pattern of isozymes in 2- versus 52-week-old rats. Sitar and Desai (1983) reported that male Sprague Dawley rats lost responsiveness with age to PB but not β-NF. McMartin et al. (1980) observed the same pattern in Sprague Dawley rats. In contrast, Baird et al. (1975) reported no decline in responsiveness of male CFN rats to PB. Birnbaum and Baird (1978) examined the effectiveness of three different inducing agents (PB, 3-MC, and PCN) in Fischer 344 rats. They observed that inducibility of microsomal benzphetamine demethylation and aniline hydroxylation decreased with age, whereas inducibility of p-nitroanisole demethylation increased with age.

Thus, despite the lack of uniformity in the literature, age clearly plays a role in the response of rats to inducing agents. The extent that age plays depends on the stain and sex of the rats used, the class of inducing agent, and the specific MFO parameter examined. As a broad generality, most rats lose responsiveness to PB induction as they age. What are the implications of age-related changes in MFO activity? One should expect changes in PK behavior of a given drug or chemical in rats. Kapetanovic et al. (1982), for example, reported that the plasma half-life was longer and plasma clearance rate lower in old (32–34 months) rats than in young (3–4 months) rats. If toxicity of a chemical depends on metabolic activation (e.g., an indirect hepatotoxin), there may well be age-related changes in sensitivity. Rikans (1984) examined age-related changes in response to various hepatotoxins and reported that allyl alcohol toxicity was more severe in middle-aged and old rats than in young-adult rats. The sensitivity of rats in acute toxicity testing varies considerably with age, and this may be due in part to age-related differences in metabolism. Hence, one should always consider the age of the rats in attempting to reproduce an experimental result.

Other Influences of MFO Activity

Age, sex, and the strain of rats used all play modulating roles in xenobiotic metabolism. Husbandry and other external events have also been shown to influence xenobiotic metabolism. The feeding regimen is a good example: Gram et al. (1970) reported almost 20 years ago that 72 hours of starvation elicited increases in cytochrome P_{450} and MFO activity. This is consistent with the authors' experience that fasting increases the sensitivity of the rat to many indirect hepatotoxins. Radzialowski and Bousquet (1968) reported that MFO activity followed a diurnal cycle that tended to mirror the diurnal cycles in plasma corticosterone levels. Hence, the time of day a rat is dosed can affect the subsequent metabolism and PK behavior of the drug or chemical under investigation. In another classic work, Fuller et al. (1972) demonstrated that cold exposure (4°C for 4 days) stimulated hepatic drug metabolism in the rat. Hence, environmental conditions can also influence MFO activity in the rat. Room conditions in a toxicity study should be carefully controlled and monitored to avoid temperature extremes that could alter the rates of test article metabolism.

Peroxisomal Proliferation

Peroxisome proliferator–activated receptors (PPARs) are ligand-activated transcription factors that are involved in regulating glucose and lipid homeostasis, inflammation, proliferation, and differentiation. The PPARs α (NR1C1), β/δ (NR1C2), and γ (NR1C3) are members of the nuclear-hormone receptor (NR) superfamily (Pyper et al., 2010). The foundation for the discovery and designation of the PPAR subfamily of nuclear receptors in the 1990s (Dreyer et al., 1992; Issemann and Green, 1990) is the cumulative work over the preceding 25 years (Cayen et al., 1982) with peroxisome proliferators (PPs), a group of structurally diverse chemicals that lower serum lipids and induce massive proliferation of peroxisomes in liver cells, with associated coordinated transcriptional activation of peroxisomal fatty acid β-oxidation system genes (Table 3.29).

Drugs affecting peroxisome proliferation generally fall into two classes: hypolipidimic drugs related in structure to clofibrate and plasticizers related in structure to di(2-ethylhexyl)phthalate. These agents cause hepatomegaly, proliferation of smooth endoplasmic reticulum with increases in MFO activity, increases in peroxisomal number with associated enzyme level changes, and changes in mitochondrial number and function with increases in certain enzyme levels. This phenomenon has been most extensively studied by Reddy and colleagues (1983) in rats and mice. The phenomenon is diagnosed microscopically by increased content of peroxisomes or biochemically by measuring the activity of peroxisomal specific enzymes, such as catalase or carnitine acetyltransferase. They also, as reported by Oesch et al. (1983), cause large increases in cytosolic epoxide hydrolase (c-EH). For example, these investigators have reported 1 week of treatment with clofibrate (200 mg/kg/r day, dietary admix) will cause a 50% increase in liver relative organ weights (from 4.1% to 6.3%) and eightfold increase in both palmitoyl-CoA transferase activity (from 11.4 to 90.0 nmol/min/mg) and c-EH activity (38–312 pmol/min/mg protein) in rats.

Bezafibrate is a hyperlipidemic drug that is a potent peroxisomal-proliferating agent. Halvorsen (1983) reported that administration of bezafibrate caused 60% increases in liver weight and total hepatic protein and a 2.4-fold increase in carnitine palmitoyl transferase activity in male Wistar rats. Watanabe et al. (1989) demonstrated that a longer dosing period (100 mg/kg for 13 weeks) can have even more dramatic effects, e.g., a 35.8-fold increase in carnitine acyltransferase. Increases were seen in other species, but (except for mice) nowhere near the increases seen in rats.

The safety of peroxisomal-proliferating agents is an issue because these agents are also liver carcinogens in rats and mice. Rats and mice are the most inducible and other species are less responsive. It would appear that rats are good models to determine if an agent causes peroxisomal proliferation, but poor models to determine if such agents are genotoxic carcinogens. These agents will almost always uniformly (100% response) cause hepatocellular tumors at the MTD in rats.

Table 3.29   Known Genes Associated with Peroxisome Proliferation Activity in the Rat

Gene Name	Aliases/Other Designations	Summary
Ppara	PPAR	PPAR α; nuclear steroid receptor; heterodimerizes with retinoid-X receptor and binds to PP response elements to initiate the transcriptional regulation of target genes
Ppard	Pparb	PPAR β, transcription factor of the NR superfamily; may mediate the intestinal expression of target genes implicated in lipid metabolism
Pparg		PPAR γ; ligand-activated transcription factor; mediates expression of genes involved in lipid metabolism
Ppargc1a	Ppargc1	Nuclear receptor coactivator; may mediate glucose homeostasis, and lipid homeostasis
Ppargc1b	Perc; PGC1beta	PPAR gamma, coactivator 1 beta

Source: PubMed Gene database, http://www.ncbi.nlm.nih.gov/genbank/, accessed June 11, 2015.

As these tumors are probably a result of the peroxisomal proliferation, the relevance of these findings to nonresponsive species is questionable. The potential carcinogenicity of peroxisomal-proliferating agents may be better assessed in a species such as the guinea pig, which Oesch et al. (1988) have shown to respond poorly to peroxisomal-proliferating agents.

The mechanism by which peroxisomal-proliferating agents cause hepatic carcinomas in rodents is a subject of some debate. One theory holds that the increase in peroxisomal number leads to an increase in the generation of reactive oxygen species that can damage DNA. The oxidative stress hypothesis has come under some question because there is little evidence to suggest that oxidative injury in and of itself is sufficient to cause genotoxic changes. An alternative explanation is that peroxisomal-proliferating agents also have promoting activity. Cattley and Popp (1989) have studied this hypothesis using WY-14643 as a model peroxisomal-proliferating agent and compared its promoting activity with that of PB. Both agents were very effective (100% response in 4–5 weeks) in promoting altered hepatic foci in diethylnitrosamine-initiated rats, but with qualitative differences. In contrast to PB, WY-14643-promoted foci were larger, did not stain for r-glutamyl-transpeptidase, and were highly ATPase deficient. The authors concluded that these differences were evidence that WY-14643 is a tumor promoter (as well as a peroxisomal-proliferating agent), but the mechanism of promotion differs from that of PB. In fact, the mechanisms of WY-14643 promotion and peroxisomal proliferation may be distinct. Whether these findings can be generalized to all peroxisomal-proliferating agents remains to be established and the role of peroxisomal proliferation in carcinogenicity needs to be more thoroughly examined. The rat is probably the species of choice for such investigations.

Flavin-Dependent Mixed Function Oxidase

Not all the microsomal MFO activity is cytochrome P_{450} dependent. There is a separate FMO that is different and distinct from the cytochrome P_{450}–dependent MFO. Dannan and Guengerich (1982) clearly identified with immunochemical techniques the presence of this enzyme in various rat tissues, including the rat liver. The role of this enzyme in drug development was reviewed by Cashman (2008). There are species- and organ-related isozymes, but the presence of more than one different isozyme in the same organ has not yet been demonstrated. There are currently no data to suggest that this enzyme is inducible in the strictest sense of the word (i.e., causes increases in specific activity). The literature is inconsistent with regard to sex-related differences. Ziegler (1998) reported that, as with cytochrome P_{450}–dependent activity, there is a greater activity in males than in female rats (approximately 5:1), while Dannan and Guengerich (1982) reported nearly equivalent activity in male and female rats. Also in rats, the highest amounts of activity are found in the liver, although in female rats, nearly equivalent amounts (on a gram tissue basis) are found in the liver and kidney (Dannan and Guengerich, 1982).

N-Octylamine (an inhibitor of cytochrome P_{450}–dependent MFO) is an allosteric activator of the hog liver FMO (the preparation traditionally used to study this enzyme), but not of the rat hepatic FMO. The FMO metabolizes chemicals containing thiol, sulfide, thioamide (secondary and tertiary), amine, hydrazine, and phosphine substituents. This enzyme requires NADPH as a cofactor and is inhibited by both carbon monoxide and SKF 525A. With these similarities to the MFO, it can be difficult to distinguish between them and determine which enzyme system is actually playing the predominant role in the metabolism of a specific chemical, especially *in vivo*. In *in vitro* preparations, however, enzyme-specific antibodies can be used to determine the relative contributions of each system. For example, Tynes and Hodgson (1985) used antibodies to the NADPH–cytochrome c reductase to demonstrate that 100% of the thiobenzamide S-oxidation is due to the FMO.

The implication of such findings is that for the aforementioned classes of chemicals, there may be two competing pathways of oxidative metabolism (in addition to the competing isozymic cytochrome P_{450} pathways). For example, Cashman (1989) reported that verapamil is oxidized by both systems in rats: via the FMO, the main metabolite is 3,4-dimethoxystyrene, and via the MFO, the main metabolite

Table 3.30 FMO Genetic Profiles in the Rat

Gene Name	Aliases/Other Designations	Summary
Fmo1	RFMO1A	Catalyzes thiobenzamide S-oxidation; has additional S- and N-oxidation activities as well
Fmo3		Flavin containing monooxygenase 3; responsible for methimazole S-oxidation and NADPH oxidation
Fmo4		Flavin containing monooxygenase 4
Fmo5		Flavin containing monooxygenase 5

Source: PubMed Gene database, http://www.ncbi.nlm.nih.gov/genbank/, accessed June 11, 2015.

is N-desmethylverapamil. As 3,4-dimethoxystyrene is the predominant metabolite, the FMO is the major enzyme involved in verapamil metabolism in the rat. Interestingly, the FMO is stereoselective for the S(+)-enantiomer of verapamil, which results in stereoselective first-pass metabolism of verapamil with shifts in the S(+)/R(–) ratio. In general, the FMO is probably stereoselective in the metabolism of many chemicals. In studying the metabolism of racemic mixtures of potential FMO substrates, one should be aware of the possibility of stereoselective first-pass metabolism. As pharmacological properties of optical isomers are frequently different, stereoselective metabolism may cause changes in pharmacological actions that do not follow changes in plasma concentrations of a drug (Table 3.30).

Epoxide Hydrolase

Epoxide hydrolases (EHs) have been detected in prokaryotes and eukaryotes ranging from plants to mammals (Fretland and Omiecinski, 2000; Hammock et al., 1997; Newman et al., 2005). In mammals, these include the soluble EH (sEH) and microsomal EH (mEH). In the rat, these enzymes are important in the study of xenobiotic metabolism and toxicity. The products of MFO metabolism of many aromatic and olefinic chemicals are highly reactive arene and alkene oxides. These are, in turn, substrates for EH, which converts them to inactive dihydrodiols. EH is thus truly protective, as many of the endogenous substrates of the MFO are oxidized to epoxides (Table 3.31).

sEH (EPHX2) in human is a bifunctional homodimeric enzyme located in both cytosol and peroxisomes with both EH and phosphatase activity. mEH (EPHX1) is membrane bound and found largely in the endoplasmic reticulum. Little is published about EPHX3 and EPHX4. The EH catalytic pocket consists of two tyrosine residues (Tyr381 and Tyr465), which activate the epoxide ring opening by Asp333. The resulting ester is then rapidly hydrolyzed (Shen, 2012). EH has been the subject of many reviews (see also the introductory chapter) such as those by Oesch (1972) and Seidegard and DePierre (1983). Highest activity is present in the liver, testis, kidney, ovary, and lung. mEH activity is induced in the rat by PB treatment, but only sparingly induced by 3-MC-type inducing agents. mEH is not affected by the more common MFO inhibitors, such as SKF-525A. In fact, some of the more common MFO inhibitors, such as metyrapone, are allosteric activators

Table 3.31 Profiles of Various EHs in the Rat

Gene Name	Aliases/Other Designations	Summary
Ephx1	mEH, MEH8	EH 1, microsomal (xenobiotic); catalyzes the hydration of a wide range of xenobiotic arene oxides
Ephx2	CEH, sEH, hsEH,	EH 2, sEH, cytoplasmic, catalyzes the hydrolysis of an epoxide to a glycol; plays a role in regulation of blood pressure
Ephx3	Abhd9	EH 3
Ephx4	Abhd7	EH 4

Source: PubMed Gene database, http://www.ncbi.nlm.nih.gov/genbank/, accessed June 11, 2015.

of mEH. Specific inhibitors of mEH include 1,1,1-trichloropropane-2,3-oxide and cyclohexane oxide. This enzyme also has some interesting substrate conformational specificity. For example, cis-stilbene oxide is hydrated 700 times more rapidly than the trans isomer. Treatment of rats with poor substrates like trans-stilbene oxide (400 mg/kg ip in corn oil) was once thought to specifically induce EH. This is true only in a relative sense, as trans-stilbene will cause slight increases in MFO activity. It, however, causes much larger increases in EH. Few, if any, chemicals induce EH without also increasing MFO activity to some extent.

If one plans on using inducing agents to study EH, they need to be aware that the time course of the response of cytochrome P_{450} and EH is different. For example, Parkinson et al. (1983b) demonstrated that following treatment of 4-week-old male Long-Evans rats with Aroclor 1254 (single intraperitoneal injection, 500 mg/kg), peak total cytochrome P_{450} increases occurred in 4 days, whereas maximal EH activity increase did not occur until about 10 days. The implications of this finding is that longer pretreatment periods may well be required than normally used in experiments with inducing agents (e.g., 3 days of treatment with PB tends to be common practice) if one wants assurance that maximal induction of both cytochrome P_{450} and EH has occurred.

As might be expected, EH follows a similar pattern of distribution as cytochrome P_{450}. Thus, the centrilobular hepatocytes contain higher amounts than those of the periportal region (Seidegard and DePierre, 1983). Some strain-related differences in rat hepatic EH have been identified. Oesch et al. (1983) examined mEH activity with styrene 7,8-oxide (a common model substrate) in 22 different rat strains. They found that the activity was highest in Sprague Dawley and lowest in Fischer 344 rats (4.3 vs. 12.7 nmol/min/mg protein). No qualitative differences were identified to account for this observation; they were due entirely to strain-related differences in the amounts of enzyme synthesized.

The effects of age and sex on mEH have been examined to some extent in the rat. Birnbaum and Baird (1979) studied EH in male CFN rats and reported that there were age-related increases in young (3 months), middle-aged (12 months), and old (27 months) rats. They also noted that induction of EH with PB also increased with age. Chengelis (1988d) reported on the age-related changes in EH in Sprague Dawley (Charles River) male and female rats. At 4 weeks of age, rats of both sexes had about the same activity (about 90 nmol/min/g tissue), but it increases dramatically in males thereafter, peaking (at 340 nmol/min/g) at 78 weeks of age. In females, activity remained relatively constant throughout their lifetime. This could result in age-related shifts in the metabolite profile for a chemical in male but not in female rats. One should be aware, therefore, that the extent of EH activity, and gender-related differences, may depend upon the age of the rats studied.

The catalytic activity of the sEH on arene oxides and other cyclic epoxides is so low that its contribution appears insignificant compared to the mEH, as well as for chemical and GST-catalyzed conjugation of reactive epoxides (Shen, 2012). Levels of this enzyme, however, increase markedly in rats treated with peroxisomal-proliferating agents (Oesch et al., 1988) and may have some utility as a marker for this effect. Inhibitors of sEH, however, show promise as potential therapeutic indications (Shen and Hammoock, 2012). In contrast, mEH is greatly increased in hepatic hyperplastic nodules and hepatomas (Seidegard and DePierre, 1982). Kizer et al. (1984) examined the induction of mEH by known carcinogens, such as 2-acetylaminofluorene, thioacetamide, and aflatoxin. They noted that 3 weeks of feeding with various carcinogens, which require metabolic activation, resulted in 4- to 10-fold increases in EH activity in Holtzman rats. They recommended that the induction of EH could be used as part of a preliminary screen for determining the carcinogenic potential of new chemical entities. In our opinion, such data are useful, but only as part of an overall package of short-term tests (including but not limited to genetic toxicology testing).

Miscellaneous Phase I Enzymes

There are, of course, a wide variety of enzyme systems that are responsible for Phase I metabolism. A short list is shown in Table 3.32.

Table 3.32 Profiles of Various Phase I Enzyme Systems in the Rat

Gene Name	Aliases/other Designations	Summary
Aadac	Aada	Arylacetamide deacetylase
Cel	Bal; Bssl	Carboxyl ester lipase
Ces1d	Ces3	Carboxylesterase 1D, enzyme that hydrolyzes long-chain fatty acids and thioesters; may play a role in lipid metabolism and/or the elimination of toxic substances
Ces1e	Ces1; Es22	Carboxylesterase 1E; enzyme that hydrolyzes long-chain fatty acids; necessary for cholesterol esterification; may be important for detoxification of certain drugs
Ces2c	rCG_57590, CESRL4, Ces2, Ces2l	Enzyme that is responsible for the hydrolysis of long-chain fatty acids and thioesters; may play a role in lipid metabolism and/or the detoxification of certain drugs
Maoa	Mao	Monoamine oxidase A; enzyme involved in the oxidative deamination of biogenic and xenobiotic amines
Maob		Monoamine oxidase B; major neurotransmitter-degrading enzyme in the CNS; degrades biogenic monoamines in the brain
Ptgs1	Cox1; Cox3; Cox-3	Prostaglandin-endoperoxide synthase 1; catalyzes the conversion of arachidonic acid products to prostaglandin; may be involved in methamphetamine neurotoxicity
Ptgs2	Cox2; COX-2	Prostaglandin-endoperoxide synthase 2; catalyzes the conversion of arachidonic acid products to prostaglandin; may play a role in response to endotoxic shock; may play a role in long-term potentiation
Lpo		Lactoperoxidase; extensively studied in multiple species; heme peroxidase family, catalyzes the oxidation of a number of inorganic and organic substrates by hydrogen peroxide
Mpo		Myeloperoxidase; myeloperoxidase family member that may contribute to hyperoxia-mediated lung injury via nitration of proteins, resulting in reactive nitrogen species; has a heme pigment
Pon1		Paraoxonase 1; A-esterase 1; PON 1; aromatic esterase 1; may protect LDL and HDL from oxidation and provide protection against atherosclerosis
Pon2		Paraoxonase 2; A-esterase 2; PON 2; aromatic esterase 2
Pon3		Paraoxonase 3; serum paraoxonase/lactonase 3; microsomal paraoxonase that has esterase activity on a broad range of substrates
Abp1	Abp	Amiloride binding protein 1 (amine oxidase, copper containing); binds amiloride and some of its derivatives; may form an epithelial-specific Na+ channel
Ahr		Aryl hydrocarbon receptor; binds 3-MC and other aromatic hydrocarbons; may play a role in bone formation
Arnt	Arnt1	Aryl hydrocarbon receptor nuclear translocator; may act as a transcription factor; mediates dioxin toxicity
Snn	Stannin	May play a role in the selective vulnerability of neuronal populations to the toxic effects of organotin compounds
Adh1	Adh; Adh1a; Adh1c	ADH 1 (class I): metabolizes a wide variety of substrates including ethanol, hydroxysteroids and lipid peroxidation products
Aldh1a1	Ahd2, Aldh1, Aldh2	Aldehyde dehydrogenase 1 family, member A1; catalyzes oxidation of aldehyde substrates to carboxylic acids; detoxifies ethanol-derived acetaldehyde
Xdh	XO, Xor, Xox-1, Xox1	Xanthine dehydrogenase, catalyzes the conversion of xanthine + NAD+ + H2O to urate + NADH + H+; may be involved in response to aluminum and aluminum toxicity

Source: PubMed Gene database, http://www.ncbi.nlm.nih.gov/genbank/, accessed June 11, 2015.

Phase II Reactions: Conjugation

Conjugation reactions or biosynthetic pathways are those that take the products of MFO, FMO, or EH and add an additional functionality to form a new chemical moiety. The oxidation of benzene to phenol is Phase I, whereas the formation of phenol sulfate is a Phase II reaction. Of course, if the molecule intrinsically has the appropriate functional group, such as the terminal carboxylic acid in benoxaprofen, it can undergo Phase II metabolism directly. In the case of benoxaprofen, it is metabolized to its 1-O acyl glucuronide (Obach, 2009). The rat has a complete set of conjugative reactions, including (in ascending order of importance) N-acetylation, amino acid (almost always either glycine or glutamate) conjugation, sulfate conjugation, GSH conjugation (thioether formation), and glucuronic acid conjugation. The reader is referred to Caldwell (1982) for a more complete description of these processes. The preference for a specific conjugation reaction is very much substrate specific. For example, Huckle et al. (1981) examined the *in vivo* metabolism of 3-phenoxybenzoic acid and found 5-hydroxy-3-phenoxybenzoic acid sulfate to be the major metabolite, accounting for >60% of the radioactivity in the urine. In contrast, Weyland and Bevan (1987) reported that the predominant conjugate metabolites of benzo(a)pyrene were thioethers (GSH conjugates), whereas sulfates accounted for only 10% of the excreted metabolites. Quantitative and qualitative differences in species also lead to further complications. In contrast to the rat, for example, the major metabolite of 3-phenoxybenzoic acid in the guinea pig is 3-phenoxybenzoic acid glucuronide (Huckle et al., 1981). The basic aspects of the classic conjugation reactions as they apply to the rat are as follows.

Amino Acid Conjugates

In general, amino acid conjugation is more highly developed in larger species, and amino acid conjugates are seldom major metabolites in rats. But there are some exceptions, for example, as described in Cooper et al. (2011) and Zhang (2009). Amino acid conjugates are also usually quite stable and not involved in further activation reactions (Table 3.33).

Acetylation

While O-acetylation of hydroxyl groups (ester formation) is theoretically possible, in the rat, this is apparently very rare, whereas acetylation of free amines to amides is a common reaction in rats. It is catalyzed by the cytosolic enzyme N-acetyl transferase, using acetyl-CoA as the cosubstrate (Table 3.34).

N-Acetyl transferase has gathered the most attention with regard to the metabolism (and activation) of carcinogenic aromatic amines and hydrazides. The acetylation of 2-aminofluorene is the first step in the activation of this chemical to the ultimate carcinogen. Interestingly, in comparison to

Table 3.33   Profiles of Various Amino Acid Conjugation Systems in the Rat

Gene Name	Aliases/Other Designations	Summary
Agxt		Alanine-glyoxylate aminotransferase; this gene encodes alanine-glyoxylate aminotransferase, which catalyzes the interconversion of L-alanine and glyoxylate to pyruvate and glycine.
Baat	kan-1, BACAT, BAT	Bile acid CoA: amino acid N-acyltransferase (glycine N-choloyltransferase), may play a role in acute phase response.
Ccbl1	Gtk; Katl	Cysteine conjugate-beta lyase, cytoplasmic.
Glyat	Ab2–132, AAc; acyl-CoA	Glycine-N-acyltransferase.
Ggt1	Ggt; Ggtp; GGLUT	Gamma-glutamyltransferase 1; required in GSH metabolism.

Source: PubMed Gene database, http://www.ncbi.nlm.nih.gov/genbank/, accessed June 11, 2015.

Table 3.34 Profiles of Various N-Acetyltransferase Enzymes in the Rat

Gene Name	Aliases/Other Designations	Summary
Nat1	Nat	N-acetyltransferase 1; enzyme that acetylates both arylamines and arylalkylamines
Nat2	Nat2a	Enzyme that acetylates only aryl amines; may have a role in carcinogenesis

Source: PubMed Gene database, http://www.ncbi.nlm.nih.gov/genbank/, accessed June 11, 2015.

other rodent species, the rat is a relatively poor acetylator. As reported by Lower and Bryan (1973), the hamster, guinea pig, and mouse have approximately 16, 10.5, and 9 times greater (*in vitro*) activity as the rat with 2-aminofluorene as the substrate. Lotlikar et al. (1967) reported the rat has relatively low aromatic amide hydroxylation capabilities; it is not detectable in uninduced rats. Highest activity (both baseline and induced) is found in the hamster. Hence, in the study of aromatic amine metabolism and toxicity, the hamster (not the rat) is the preferred species.

Depending on substituents, hydrazines can have a number of effects. Of most concern, however, are carcinogenicity and hepatotoxicity. Many hydrazines, hydrazones, and hydrazides have caused cancer in laboratory animals (Toth, 1979). McKennis et al. (1959) first demonstrated (in rabbits) that acetylhydrazine is the final common reactive metabolite in the hepatotoxicity of both hydrazine and isoniazid. This work was further developed using the rat as a model by Timbrell et al. (1980). Single doses of acetylhydrazine (30 mg/kg) or acetylisoniazid (200 mg/kg) produced liver necrosis in rats. Isoniazid itself, however, is poorly hepatotoxic in the rat, an observation that is consistent with the observation that rats have relatively poor N-acetyltransferase activity. Rats must be pretreated with PB and then aggressively treated with isoniazid (100 mg/kg every hour for 6 hours) to develop hepatic damage. Thus, rats are apparently poor models to study hydrazine toxicity, but are good models for the study of the metabolism and toxicity of these chemicals once they are acetylated.

Sulfate Conjugates

Sulfate ester formation (e.g., phenol sulfate formation from phenol) is catalyzed by the cytosolic enzyme PAPS-sulfotransferase using PAPS as the cosubstrate. The reaction has important toxicological involvements. For example, N-sulfo-acetyl-2-aminofluorene is the ultimate carcinogen formed from 2-acetyl-aminofluorene, involving a reaction catalyzed by phenolic-specific sulfotransferase. Generally, the availability of sulfate to be activated (PAPS formulation) is the rate-limiting step (Weinshilbuorn, 1990). Agents that depress PAPS formation also depress sulfate formation (Hjelle et al., 1985) in the rat. Chemicals with free phenolic hydroxyl groups are the most common substrates. Hence, PAPS-sulfotransferase and UGT usually compete for the same substrate (e.g., phenol), and both conjugates are frequently found together. The one that predominates will depend on the specific chemical, dose of parent chemical, and metabolic state of the rat. As a broad generalization, because of K_m differences between PAPS-sulfotransferase and UGTs, sulfates tend to predominate at lower dosages, whereas the percentage of glucuronides increases with increased dose. PAPS-sulfotransferases exist as a collection of enzymes, some with broad and some with narrow substrate specificity (Mulder, 1986) (Table 3.35).

Steroid hormones are the natural endogenous substrates for a specific class of sulfotransferases. As there are sex-related differences in steroidal hormones, it is not surprising that the rat displays considerable sex-related variation in sulfotransferases involved in steroid metabolism (as reviewed by Mulder, 1986). Common aryl sulfotransferase (p-nitrophenol) activity is 2–3 times higher in the male than female rat (Matsui and Watanabe, 1982). As a matter of course, sulfotransferases now tend to be classified as phenol sulfotransferase and steroid sulfotransferases (STs). Sekura and Jakoby (1979) isolated from rats two closely related phenol STs with activity toward a wide variety of xenobiotic

Table 3.35 Profiles of Various Sulfotransferase Enzymes in the Rat

Gene Name	Aliases/Other Designations	Summary
Sult1a1	Stm; Stp1; ASTIV; Mx-ST; PST-1; St1a1; Sult1a3	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1
Sult2a1	STa; St2; Sth2; Smp-2; St2a1	Sulfotransferase family 2A, DHEA preferring, member 1; catalyzes the metabolic activation of potent carcinogenic polycyclic arylmethanols
Sult1e1	Ste; ST1E1; ESTSUL	Sulfotransferase family 1E, estrogen preferring, member 1; isozyme that catalyzes the sulfation of estrogens, including beta-estradiol and estrone, but not of DHEA, pregnenolone, cortisol or testosterone; plays a role in estrogen metabolism
Sult1b1	ST1B1	Sulfotransferase family, cytosolic, 1B, member 1; an enzyme that catalyzes the transfer of sulfate groups onto various tyrosine and 3,4-dihydroxyphenylalanine (dopa) isomers
Sult2a6	Smp2a; St2a2; RATSMP2A; RATSMP2B; Sult2al1; Sult2a4l1	Expression reduced during the androgen-sensitive state of young adulthood and increased during the androgen-insensitive phases of prepuberty and senescence
Sult2b1	ST2B1	Sulfotransferase family, cytosolic, 2B, member 1
Sult1c3	Stp2; St1c1; HAST-I; Sult1a2; Sult1c1	Sulfotransferase family, cytosolic, 1C, member 3; male-specific sulfotransferase that catalyzes the sulfation of N-hydroxy-2-acetylaminofluorene; plays a role in the bioactivation of N-hydroxyarylamines in the liver
Sult1c2	ST1C2	Sulfotransferase family, cytosolic, 1C, member 2; catalyzes the sulfation of p-nitrophenol; localized to lysosomes
Sult1c2a	ST1C2A; Sult1c1	Sulfotransferase family, cytosolic, 1C, member 2a
Sult1d1	ST1D1; Sultn; Sult-n	Sulfotransferase family 1D, member 1; may act as a tyrosine-ester sulfotransferase
Sult4a1	ST4A1; Sultx3	Sulfotransferase family 4A, member 1; brain-specific isoform of sulfotransferase enzyme; involved in the sulfation of several drugs, neurotransmitters, and hormones (including T3, T4, and estrone)
Tst	RHODAN	Thiosulfate sulfotransferase, mitochondrial matrix enzyme; may play a role in cyanide detoxification and the formation of iron–sulfur complexes

Source: PubMed Gene database, http://www.ncbi.nlm.nih.gov/genbank/, accessed June 11, 2015.

phenolic chemicals, such as phenol, O-naphthol, and p-nitrophenol. Maas et al. (1982) studied phenol sulfotransferase using 3-methoxy-4-hydroxyphenol glycol as the substrate. While there are measurable amounts of activity in the brain and kidney, the activity in the liver is two orders of magnitude greater and would by far make the greatest contribution to sulfate formation from xenobiotics. Hepatic activity peaks at about 10 weeks of age and is somewhat induced by DEX (1 mmol/kg for 2 days). There are some slight strain-related differences in activity, but generally it falls between 25 and 50 units/mg cytosolic protein. Chengelis (1988d) examined age- and sex-related differences in Sprague Dawley rats using p-naphthol as a substrate. No consistent sex-related differences were identified; however, males did have greater activity at 12 and 26 weeks of age, and at no point did females have statistically significant greater activity than males. Activity in both sexes peaked at 78 weeks of age. As UGT peaks at an earlier time point, the ratio of sulfate to glucuronide conjugates may also change as a function of age. In contrast to Sprague Dawley rats, 5-month-old Fischer 344 male rats had twice the *in vitro* activity with acetaminophen (phenol transferase) than females, whereas with glycol lithocholic acid, females had five times the activity than males (Galinsky et al., 1986). Using a relatively small dose (30 mg/kg, which is not hepatotoxic), these authors observed an age-related decrease in the sulfate to glucuronide ratio in male rats. This was confirmed with a higher dose (Galinsky et al., 1986). The reasons for the lack of consistent reportedly sex-related differences for the phenol sulfotransferase are unclear, but are likely due to strain, age, and methodology (e.g., model substrate).

As mentioned, there are clear sex-related differences in the STs. Female rat liver cytosol contains at least three sulfotransferases that transform a variety of hydroxymethyl polyaromatics (e.g., 5-hydroxy-methylchrysene) to sulfates that are potent mutagens (Ogura et al., 1990). This is an interesting exception to the "rule" that conjugates are inactive end products. The dominant isozyme in this class had no activity with p-nitrophenol, but was competitively inhibited by dehydroepi-androsterone (DHEA), but not 2,6-dichloro-4-nitrophenol. Interestingly, according to Ogura et al. (1990), neither hydroxymethylarenes nor methylarenes are heptocarcinogens in rats because of the high activity of hepatic GST.

In summary, the rat has an appreciable capability to form organic (ethereal) sulfates. As the rat has a high tendency to form phenolic chemicals when given aromatic-fused ring structures, one can almost always expect sulfate (and glucuronide)-containing metabolites to be formed in the rat. Activity is classified (by isozymic specificity) as either phenolic or STs. Both, however, are capable of activity toward xenobiotics, and the products can sometimes be toxic "activated metabolites."

Glucuronide Conjugates

UGTs are microsomal enzymes that catalyze the formation of glucuronide conjugates using UDP (UDP-glucuronosyl) as the cosubstrate. UGTs dominate Phase II processes just as P_{450}s dominate Phase I reactions, with UGT2B7 being the predominate isozyme in humans (Table 3.36).

In general, glucuronides are usually inactive end products, but this is not always the case. 1-O glucuronides are particularly unstable and have a number of products (Janssen et al., 1982). They can be hydrolyzed to the aglycone nonenzymatically in base. If the alpha carboxylic acid is substituted, the isomer will rearrange. The glucuronic acid group can also rearrange to the 2, 3, or 4

Table 3.36   Profiles of Various UGTs in the Rat

Gene Name	Aliases/Other Designations	Summary
Ugt1a1		UGT 1 family, polypeptide A1, catalyzes the glucuronidation of bilirubin; plays a role in bilirubin conjugation and excretion
Ugt1a5	Ugt1; Ugt1a6; UDPGT 1–5	UGT 1 family, polypeptide A5
Ugt1a6	Ugt1; Udpgt; Ugt1a7; UDPGT 1–6	UGT 1 family, polypeptide A6, catalyzes the glucuronidation of 4-nitrophenol
Ugt1a8	Ugt1; Ugt1a9; UDPGT 1–8	UDP glycosyltransferase 1 family, polypeptide A8, UGT; enzyme of the glucuronidation pathway
Ugt1a9	Ugt1a10; Ugt1a11	UGT 1 family, polypeptide A9
Ugt2a1	Ugt2a1p	UGT 2 family, polypeptide A1, enzyme important for the elimination of potentially toxic compounds; found in the olfactory epithelium and may be involved with olfaction
Ugt2a3	RGD1308444	UGT 2 family, polypeptide A3
Ugt2b		UDP glycosyltransferase 2 family, polypeptide B, enzyme involved in conjugating lipophilic aglycon substrates with glucuronic acid
Ugt2b1	2B1; Udpgtr2; Ugt2b17	UGT 2 family, polypeptide B1, catalyzes the transfer of glucuronate from UDP-glucuronate to beta-D-glucuronoside; may play a role in drug metabolism
Ugt2b2	None	UGT 2 family, polypeptide B2, catalyzes the transfer of glucuronate from UDP-glucuronate to thyroid hormones
Ugt2b15	Ugt2b12, Ugt2b36, Ugt2b4	UGT 2 family, polypeptide B15, enzyme that catalyzes the glucuronidation of monoterpenoid alcohols; involved in the breakdown of potentially toxic compounds
Ugt2b17	Udpgt; Rlug38; Ugt2b3; Ugt2b5; Udpgtr-3; UDPGT 2B5	UGT 2 family, polypeptide B17, enzyme that glucuronidates testosterone, dihydrotestosterone, and beta-estradiol
Ugt2b7	Ugt2b8; UDPGT 2B7	UGT 2 family, polypeptide B7

Source: PubMed Gene database, http://www.ncbi.nlm.nih.gov/genbank/, accessed June 11, 2015.

glucuronic acid esters in both base (even at pH 7.4) and UV. Smith et al. (1986b) have demonstrated that glucuronidation of zomepirac results in a reactive chemical moiety that binds to macromolecules. An excellent summary on this issue was written by Wainhaus (2005).

Generally, the generation of glucuronic acid is considered the rate-limiting step in the overall reaction. Gregus et al. (1988) have demonstrated that treatments that decrease UDP-glucuronate also decrease glucuronide formation. Large numbers of functional groups are capable of being glucuronidated, including hydroxyls, carboxyls, primary amines, and thiols to form ethers or esters (Burchell and Coughtrie, 1989; Caldwell, 1982). As with the sulfotransferases, different types of phenolic substrates are used to phenotype or characterize the different isozymes. UGT in rat has been extensively studied. Known molecular weights vary from 52,000–56,000 Da. Isozymes are divided between those that have primary activity against xenobiotics (4-nitrophenol, morphine, phenol, and 4-hychroxyphenol UGT) and those that have primary activity against endogenous substrates (17β-hydroxysteroids, 3α-hydroxysteroids, bilirubin, and estrone UGT). In general, isozymes that prefer xenobiotic substrates have broader substrate specificity than those that prefer endogenous substrates (Chowdhury et al., 1986; Tephly et al., 1988). The activity of this system is particularly important in the rat. Glucuronides are actively transported and excreted in the bile, and the rat has an extremely efficient biliary excretion system. Enterohepatic circulation of xenobiotics and metabolites is always a factor to consider in rats. Rodents are considered good biliary excretors compared to humans, and this can be reflected in species differences in the extent of fecal excretion and enterohepatic recycling. For example, indomethacin is so highly ulcerogenic in the rat as opposed to the guinea pig (Mariani and Bonanomi, 1978) because the enterohepatic circulation of indomethacin results in greater exposure of the intestinal mucosa. Despite the efficiency of this species in biliary excretion, UGT activity in the rat is not distinctively high. Astrom et al. (1987) examined the activity of microsomal preparations against 1-naphthol for various species (rat, mouse, guinea pig, and hamster). There were not large differences between species, mean activity from 5.9 to 12 nmol/mg/min. Guinea pigs had the highest activity. Boutin et al. (1984) examined a much larger number of substrates in five different species. Again, there was nothing either quantitatively or qualitatively distinctive about the activity in rats, and activity in guinea pigs tended to have the highest activity.

In the rat liver, UGT is also a highly inducible enzyme, particularly those with isozyme activity toward exogenous substrates. It has been conclusively known since the early 1970s that PB and polycyclic aromatic hydrocarbons induce not only P_{450} but UGT as well (Vainio et al., 1974). Mulder (1970) reported that PB increased p-nitrophenol UGT activity nearly 90%. Bock et al. (1988) have isolated and characterized the 3-MC-inducible form of (phenol), UGT, and found that 3-MC (40 mg/kg ip in olive oil; single treatment) increased microsomal UGT activity with 1-naphthol almost four times, whereas having no effect with testosterone as the substrate. As with the MFO, different types of inducers induce different enzymes. For a complete review of the inducible characteristics of UGT, the reader is referred to Burchell and Coughtrie (1989). The induction of UGT, however, is not a common mammalian characteristic and may be distinctive to the rat. Astrom et al. (1987) reported that 3-MC caused a 6 times increase in microsomal UGT activity (with 1-naphthol) in male rats, had at best modest effects in guinea pigs and C57BL/6 mice (17 and 20% increase, respectively), and caused actual decreases in hamsters and DBA/2 mice (27 and 21%, respectively). The different isozymes also have different lobular distributions in the rat liver. As could be expected, those that prefer xenobiotic substrates (p-nitrophenol) have a distribution similar to the MFO, with the largest amount in the centrilobular region. 3α- and 17β-Hydroxysteroid UGTs were uniformly spread across the lobule, with equal amounts in the centrilobular, midzonal, and periportal regions (Knapp et al., 1988). Induced UGTs can also metabolize endogenous substrates. UGTs metabolize thyroid hormones and facilitate their biliary excretion. Induction of UGTs by microsomal enzyme inducers can result in increased glucuronidation of thyroid hormones, resulting in a decrease in plasma thyroid hormones, with a feedback increase in TSH (Richardson and Klaassen, 2010).

As reviewed by Mulder (1986), male rats generally have higher hepatic UGT activity than female rats. There are exceptions, particularly with endogenous substrates; females, for example, have higher 17,6-steroid UGT activity than males. Mulder suggests that differences between sexes were quite variable and dependent on assay conditions. This was confirmed by Astrom et al. (1987), who demonstrated that there was no difference in the microsomal activity toward 1-naphthol unless the preparations were treated with a detergent. This situation may also be complicated because there are sex-related differences in age-related changes. Chengelis (1988d) reported that microsomal p-nitrophenol UGT in Sprague Dawley rats peaked in both sexes at 39 weeks of age, but males had significantly higher activity at 12, 26, and 39 weeks of age. There were no sex-related differences after 52 weeks of age, and activity fell off precipitously in senescent rats. In contrast, while Galinsky et al. (1986) identified no age-related changes in UGT activity with variety of substrates, males (also 5 months old) had consistently higher activity than females (also 5 months old). Borghoff et al. (1988) also reported decreases in UGT activity in male Fischer 344 rats. Using 4,4'-thiobis(6-t-butyl-m-cresol) as a model, they detected a decrease in the amount of glucuronide formed, decreased microsomal activity in vivo, and age-related decreases in UGT as substrate. Thus, gender as well as age-related changes could play a role in glucuronide formation.

Developmental changes in UGT have been characterized in the rat, as reviewed by Burchell and Coughtrie (1988). For the isozymes most involved in xenobiotic metabolism, there are two developmental clusters. Activities toward planar phenolic structures (e.g., 4-nitrophenol) develop prior to birth and reach a perinatal maximum 2 days postpartum. Activity toward more bulky molecules (morphine, chloramphenicol) does not appear until after birth and reaches a perinatal maximum 25–30 days postpartum. These changes should be kept in mind when one is examining the metabolism or toxicity of xenobiotics in neonatal animals.

Considerable strain variation is demonstrated by UGT. The Gunn rat, for example, is an inbred substrain of the Wistar rat that has lifelong hyperbilirubinemia because of low UGT activity with bilirubin. The Gunn rat has very low activity with a variety of planar phenolic substrates, and activity is not inducible by 3-MC (Burchell and Coughtrie, 1989), but almost the expected level of activity with testosterone and similar substrates. Boutin et al. (1987) reported that the microsomal activity of Wistar rats with 1-naphthol was 34 nmol/min/mg and with testosterone was 6.4 nmol/min/mg. In Gunn rats, these activities were 113 and 4.3 nmol/min/mg. Other genetic variations have been described in Wistar rats; approximately 50%–60% have high (whereas 40%–50% have low) 3-hydroxysteroid UGT (Knapp et al., 1988). While such differences obviously will alter the metabolism of some endogenous chemicals, the implications for the metabolism of xenobiotics are unclear. One should be aware, however, that different Wistar rats may produce unpredictably different metabolic profiles with some chemicals.

Glutathione and the Glutathione S-Transferase(s)

This system is probably the most important conjugation system in the rat liver with regard to "inactivation" of activated metabolites. GSH is a tripeptide (y-glutamyl cystinyl-glycine) that accounts for over 90% of nonprotein thiols in the liver (Levine, 1983). From a xenobiotic viewpoint, the cystinyl group is the most important. GSH readily reacts (nucleophilic attack) with arene oxides, epoxides, and aryl and alkyl halides to form thioethers. The oxidation of reduced GSH to the dithiol (GSSG) also plays an important role in protection against oxidative stress (Boyd, 1980). As reviewed by Kaplowitz et al. (1985), the transport, synthesis, and activity of the GSH redox cycle (GSH reductase, GSH peroxidase) act to keep GSH at a fairly constant level. The concentration of (reduced) GSH in rat liver tends to range between 4 and 8 mmol/g liver (e.g., see Chengelis, 1988b,d; Igarashi et al., 1983; Moron et al., 1979) and is present in much larger amounts than the oxidized disulfide form by an approximately 20:1 ratio (Igarashi et al., 1983). No consistent sex-related differences in GSH content have been reported. The effects of different inducing agents on hepatic GSH levels have not been thoroughly explored, but at least one author has reported that treating rats with

PB results in increases in GSH (Utley and Mehendale, 1989). Within the hepatocyte, GSH exists in different distinct pools (Levine, 1983). Reed (1990) recently reviewed the toxicological implication of mitochondrial, as opposed to cytosolic, GSH. About 10%–15% of hepatic GSH in the rat is in the mitochondrial fraction. For example, the cytotoxic effect of ethacrynic acid in isolated hepatocytes correlated with depletion of mitochondrial not cytosolic GSH (Table 3.37).

While GSH is generally considered a cosubstrate in a synthetic reaction (for every mole of conjugate, 1 mole of xenobiotic and 1 mole of GSH are consumed), GSH can also play a catalytic role. In the rat (and probably other species as well), GSH is involved in the metabolism of formaldehyde (Levine, 1983) and dihalomethanes (Ahmed and Anders, 1978). In both cases, the first step is the formation of a conjugate that is then the substrate for a final enzyme. For example, bromoethane is first conjugated (with release of one halide ion) to an s-halomethyl-GSH intermediate; this is further metabolized by a cytosolic enzyme to form formaldehyde, another bromide ion, and intact GSH.

Additionally, while GSH conjugates are generally inactive end products, there are numerous examples of GSH conjugates that are toxic or "activated metabolites in and of themselves" (Caldwell, 1982; Picket and Lu, 1989). Cysteine-S conjugates of tetrachloroethylene, chlorotrifluorethylene, and other chlorofluorocarbons are potent nephrotoxins in rats. *In vitro* GSH conjugates of ethylene dibromide lead to the formation of an active metabolite (as measured by DNA adduct formation). The same results were obtained using both purified enzymes and isolated hepatocytes (human and rat), suggesting that the rat is a good predictive model for these types of activation reactions. Efavirenz was found to cause renal tubule toxicity in rats but not in monkeys or humans (Baillie and Rettie, 2011). The drug was extensively metabolized in all three species, but only the rat produced a cysteinylglycine conjugate from a precursor GSH conjugate. It was determined that the basis for the species difference in efavirenz renal toxicity is likely related to species differences in GST activity between rats, monkeys, and humans.

Table 3.37   Profiles of Various GSTs in the Rat

Gene Name	Aliases/Other Designations	Summary
Mgst1		Microsomal GST 1; enzyme that catalyzes the transfer of GSH onto hydrophobic substrates
Mgst2		Microsomal GST 2
Mgst3		Microsomal GST 3
Gsta3	Yc1; Gsta1; Gsta5	GST A3; may play a role in drug resistance and carcinogenesis
Gsta4	GST 8–8; GST A4–4; GST K; GST Yk	GST alpha 4
Gstk1	GST13–13; GSTkappa	GST kappa 1, member of a family of enzymes that catalyze the conjugation of GSH to a variety of electrophiles
Gstm1	GSTA3, GST 3–3; GST Yb1; GSTM1–1	GST mu 1; multifunctional enzyme thought to have scavenger function; able to neutralize harmful metabolites from foreign substances
Gstm2	GSTA4; GST 4–4; GST Yb2; GSTM2–2	GST mu 2; subunit of GST, which may play a role in xenobiotic metabolism and response to oxidative stress
Gstm3	Gstm4; GstYb4	GST mu 3
Gstm4		GST mu 4
Gstm5		GST, mu 5; involved in conjugating GSH with a variety of electrophilic substrates
Gsto1	SPG-R, GSTO 1–1; GSTO-1; MMA(V) reductase	GST omega 1, GSH-dependent dehydroascorbate reductase
Gsto2	GSTO 2–2, MMA(V) reductase	GST omega 2
Gstp1	Gst3; Gstp; GST-P; Gstp2	GST pi 1; may play a role in drug and xenobiotic metabolism

Source: PubMed Gene database, http://www.ncbi.nlm.nih.gov/genbank/, accessed June 11, 2015.

GSH conjugates are both actively further metabolized and excreted (Levine, 1983) in the rat and most species. As reviewed by Picket and Lu (1989), GSH conjugates undergo stepwise cleavage (first by y-glutamyltranspeptidase and then by cysteinylglycine dipeptidase) of two amino acid residues to a cysteine conjugate. This is a breakpoint; the cysteinyl conjugate is either acetylated (by N-acetyl transferase) to a mercapturic acid or cleaved to a free thiol (by the enzyme cysteine conjugate 6-lyase). This latter reaction has been shown to have important toxicological implications in the rat in that the free thiols formed, for example, are responsible for the nephrotoxicity discussed in the previous paragraph.

The reactions between GSH and activated xenobiotics are catalyzed by the enzyme GST. The structure and molecular biology of this enzyme have been thoroughly explored, especially in the rat (Boyer, 1989; Keen and Jakoby, 1978; Picket and Lu, 1989). GST isoenzymes are encoded by three separate families of genes (designated cytosolic, microsomal, and mitochondrial transferases), with distinct evolutionary origins, that provide mammalian species with protection against electrophiles and oxidative stressors in the environment (Higgins and Hayes 2011). They represent different heterodimeric combinations of different monomeric structures. This organization contributes to the very broad substrate specificity displayed by the rat cytosolic GST (Keen and Jacoby, 1978). A brief survey of the literature indicates that the most common model substrates used for examining total activity are 1-chloro-2,4-dinitrobenzene (CDNB), p-nitrobenzyl chloride, and 1,2-dichloro-4-nitrobenzene. These have been used probably because they are good substrates for the wide spectrum of isozymes.

The total activity of cytosolic GST is high in the rat; as much as 5% of the cytosolic protein in the rat may be GST. As reviewed elsewhere (Boyer, 1989; Picket and Lu, 1989), these macromolecules also have binding and transport rules distinct from the role they play in xenobiotic metabolism. They are extremely stable; rat cytosolic preparations can be left at refrigerator temperatures for several days with no change in GST activity. Rarely is GST activity the rate-limiting step in GSH conjugation. Generally, the amount of GSH is rate limiting, and GSH depletion has important metabolic and toxicological consequences. For a variety of metabolically activated hepatotoxins, cell damage does not occur until the metabolism results in GSH depletion. The classic example is acetaminophen toxicity, which causes a potentially fatal, hepatic centrilobular necrosis when taken in overdose (James et al., 2003). Via a very minor metabolic path with normal dosing, acetaminophen was metabolically activated by cytochrome P_{450} enzymes to a reactive quinone metabolite, N-acetyl-p-benzoquinone imine. GSH conjugates the quinone until it is depleted, at which time the quinone is free to covalently bind to protein, primarily in the centrilobular cells of the liver where the quinone is formed. Repletion of the GSH with N-acetylcysteine protects the liver against damage. If not, death results days or weeks afterward due to liver failure if a transplant cannot be performed.

In comparison to other species, the activity of GST in the rat is by no means the highest. Astrom et al. (1987) reported that the hamster, mouse, and guinea pig all had greater activity than the rat with CDNB as the substrate. Gregus et al. (1985) have reported that species-related differences in GST can be highly substrate dependent. Perhaps the best way to summarize GST activity in the rat in comparison to other species is that the rat may not always have the highest activity, but it generally has good activity with a wider range of substrates than most species. Rodents, in general, tend to have higher activity than nonrodent. Down and Chasseaud (1979), for example, compared GST activity in cytosolic preparations in the rat, baboon, and rhesus monkey with four different substrates; the rat had from 5 to 20 times the activity depending on substrate. As discussed elsewhere (see Chapter 9), primates have higher amounts of epoxide hydratase than rats. As GST and EH serve the same "deactivating" role in the metabolism of potential toxins, this difference does not necessarily result in phylogenetic differences in toxicity, but can result in predictable differences in metabolites formed.

Hepatic GST in the rat is influenced by a variety of factors such as age, sex, strain, and diet. As reviewed by Mulder (1986), data on sex-related differences have been highly complex

and often dependent on the substrate examined and the age of the rat. Chengelis (1988d) examined age- and sex-related changes in hepatic GST in Sprague Dawley rats with two substrates (p-nitrobenzyl chloride and CDNB). With both, activity was greater in males than females only in rats 26 through 78 weeks of age; there were no significant differences in young (4–12 weeks) or senescent rats (104 weeks of age). In both sexes, activity peaked at 52 weeks of age. This is consistent with the earlier findings of Igarashi et al. (1983), who also demonstrated that there were no sex-related differences in GST activity in young-adult rats. The different monomeric subunits are evidently under different controlling influences that complicate generalizations about sex-related differences in the rat. For example, Tasman et al. (1981) reported that orchidectomy resulted in decreased activity toward three substrates, but an increase toward a fourth. James and Pheasant (1978) reported that GST activity is very low in neonatal rats. Given the complex tapestry of such controls, it is sometimes difficult to predict the extent GSH conjugation will play in the metabolism of a specific chemical in the rat, and the presence or (absence) thereof will always have to be confirmed experimentally.

In the rat, GST is also an inducible enzyme, although (as could be expected) the different monomers are differentially affected by different agents. Baars et al. (1978) examined the effect of PB, 3-MC, and TCDD (2,3,7,8-Tetrachlorodibenzo[b,e][1,4]dioxin) treatment of rats on cytosolic GST activity with three different substrates (styrene oxide, 1,2-butylene oxide, and CDNB). In general, while all three treatments caused increases in activity with all three substrates, 3-MC appeared to be the best inducing agents. Down and Chasseaud (1979) reported that DDT was a better inducing agent than PB and that neither agent increased GST activity in nonhuman primates. Astrom et al. (1987) compared the inducing effect of 3-MC on GST activity (with CDNB) in four different rodent species; increases were induced in the rat and hamster, but not the mouse or guinea pig. In general, induction of GST activity is a trait seen in relatively few species, including the rat, where only 3-MC and DDT appear to induce at best relatively modest increases in this type of activity.

Inhibition of GST can occur; for example, Aitio and Bend (1979) reported on the inhibitory effect of various common solvents (e.g., ethanol). Dierickx (1982) reported on the inhibitory effect of divalent metal ions (such as $HgCl_2$). Jakobson et al. (1979) reported that oxidized GSH (GSSG) and S-octylglutathione are effective inhibitors of GST. As a tool in the study of xenobiotic metabolism, inhibition of GST in the rat has not been widely used. This is probably because the enzyme has such high activity that partial inhibition has little effect on actual conjugate formation. As mentioned, GSH availability tends to be the limiting factor in GSH adduct formation. Most investigators have relied on GSH depletion not GST inhibition to study the involvement of this system in metabolism and toxicity. Diethyl malonate is a substrate for GST (Early and Schnell, 1972) that rapidly depletes hepatic GSH and, therefore, is frequently used in studies that require GSH depletion. For example, Chengelis (1988b) reports that diethyl malonate (640 mg/kg ip, neat) resulted in an 86% decrease in hepatic GSH content in 60 min.

Not all the GST isozymes are present in the rat liver under all circumstances. Ito and colleagues (1989) have identified a placental form that is expressed in altered hepatic or preneoplastic foci in rat liver. They have demonstrated that this is an excellent marker for these types of changes and has the advantages of being detectable (by immunohistochemical staining) in formalin-fixed tissues.

There is also ample evidence that there is a distinct microsomal GST in the rat (Boyer et al., 1989). While Boyer has suggested that this enzyme plays an important role in xenobiotic metabolism, the full implications of this enzyme in metabolism and toxicity in the rat remain to be clarified.

Other Enzymes

As mentioned in the introduction to this section, all species have a wide variety of esterases (Leinweber, 1987). As a rule of thumb, any ester given to a rat will be rapidly hydrolyzed (Table 3.38).

Table 3.38 Profiles of Various Esterases in the Rat

Gene Name	Aliases/Other Designations	Summary
Aadac	Aada	Arylacetamide deacetylase
Cel	Bal; Bssl	Carboxyl ester lipase
Esd		FGH; S-formylglutathione hydrolase; esterase D/ formylglutathione hydrolase, esterase D
Uchl1	cb358	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thioesterase)
Gzmb	RNKP-1	Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1); serine proteinase found in the duodenum
Uchl3	RGD1561196	Ubiquitin carboxyl-terminal esterase L3 (ubiquitin thioesterase)
Ces1d	Ces3	Carboxylesterase 1D, enzyme that hydrolyzes long-chain fatty acids and thioesters; may play a role in lipid metabolism and/or the elimination of toxic substances
Ces1e	Ces1; Es22	Carboxylesterase 1E; enzyme that hydrolyzes long-chain fatty acids; necessary for cholesterol esterification; may be important for detoxification of certain drugs
Ces2c	rCG_57590, CESRL4, Ces2, Ces2l	Enzyme that is responsible for the hydrolysis of long-chain fatty acids and thioesters; may play a role in lipid metabolism and/ or the detoxification of certain drugs
Ces5a	Ces5; Ces7	Carboxylesterase 5A

Source: PubMed Gene database, http://www.ncbi.nlm.nih.gov/genbank/, accessed June 11, 2015.

Like all other species, the rat possesses alcohol dehydrogenase (ADH), which metabolizes an alcohol to an aldehyde, which can be highly reactive. The ADHs are cytoplasmic, dimeric, Zn-containing enzymes (Testa and Krämer, 2010). They are widely distributed in the body, but their highest activities are found in barriers of entry, such as stomach and liver, and in organs that need special protection such as the brain. The preferred substrates are primary alcohols (such as ethanol) but are also able to oxidize secondary alcohols and even some aldehydes and to reduce aldehydes and ketones (Table 3.39).

Fortunately, the rat (as well as humans) contains an abundance of aldehyde dehydrogenases and oxidases, as shown in Table 3.40, which metabolize a wide variety of aliphatic and aromatic aldehydes to various end products, generally a carboxylic acid. They are widely distributed in the body and occur as cytosolic, microsomal, or mitochondrial enzymes. Aldehydes are toxic and interruption of their oxidation by either an alcohol overdose or coadministration of an aldehyde dehydrogenase inhibitor such as disulfiram can be fatal. The disulfiram drug Antabuse (Hardman et al., 1995) is used as a treatment for alcoholism.

Table 3.39 Profiles of Various ADHs in the Rat

Gene Name	Aliases/Other Designations	Summary
Adh1	Adh; Adh1a; Adh1c	ADH 1 (class I): metabolizes a wide variety of substrates including ethanol, hydroxysteroids, and lipid peroxidation products
Adh4	ADH-1; Ac1002	ADH 4 (class II),
Hsd17b1	17BHD1	Catalyzes the conversion between 17 beta-hydroxy- and 17-ketosteroids; plays a role in estradiol biosynthesis
Hsd17b2		17-beta hydroxysteroid enzyme that regulates the biological activity of sex hormones, including estrogen and androgens
Hsd17b3		Catalyzes the final step of testosterone biosynthesis in the testis

Source: PubMed Gene database, http://www.ncbi.nlm.nih.gov/genbank/, accessed June 11, 2015.

Table 3.40   Profiles of Various Aldehyde Dehydrogenases in the Rat

Gene Name	Aliases/Other Designations	Summary
Aldh1a1	Ahd2, Aldh1, Aldh2	Aldehyde dehydrogenase 1 family, member A1; catalyzes oxidation of aldehyde substrates to carboxylic acids; detoxifies ethanol-derived acetaldehyde
Aldh1a2	Raldh-2	Aldehyde dehydrogenase 1 family, member A2; enzyme necessary for the production of retinoic acid
Aldh1a3	Aldh6	Aldehyde dehydrogenase 1 family, member A3
Aldh1b1		Aldehyde dehydrogenase 1 family, member B1; also aldehyde dehydrogenase 1B1; aldehyde dehydrogenase X, mitochondrial
Aldh2		Aldehyde dehydrogenase 2 family (mitochondrial); also ALDH class 2; ALDH-E2; ALDH1
Aldh3a1	AHDC; Aldh; Ahd-c; Aldh3	Aldehyde dehydrogenase 3 family, member A1; expressed during hepatocarcinogenesis
Aldh3a2	Aldh4; FALDH	Aldehyde dehydrogenase 3 family, member A2
Aldh3b1		Aldehyde dehydrogenase 3 family, member B1
Aldh3b2		Aldehyde dehydrogenase 3 family, member B2
Aldh5a1	Ssadh	Aldehyde dehydrogenase 5 family, member A1
Aldh6a1	Mmsdh	Aldehyde dehydrogenase 6 family, member A1
Aldh7a1	antiquitin	Aldehyde dehydrogenase 7 family, member A1
Aldh8a1		Aldehyde dehydrogenase 8 family, member A1
Aldh9a1	Tmaba-dh	Aldehyde dehydrogenase 9 family, member A1; catalyzes the conversion of gamma-trimethylaminobutyraldehyde to gamma-butyrobetaine in carnitine biosynthesis
Tas1r2	Tr2; Gpr71; Aldh4a1	Taste receptor, type 1, member 2
Aox1		Aldehyde oxidase 1

Source: PubMed Gene database, http://www.ncbi.nlm.nih.gov/genbank/, accessed June 11, 2015.

Extrahepatic Xenobiotic Metabolism

That various tissues other than liver possess xenobiotic-metabolizing ability has been recognized for quite some time (Litterst et al., 1975). In general, given the relatively small size of these organs and the relatively low activity of the salient enzymes (compared to the liver), extrahepatic metabolism rarely plays a major role in the overall disposition of xenobiotics. For example, Litterst et al. (1975) noted that the lungs had only 7% of the cytochrome P_{450} as the liver on a microsomal basis and only 35% of the microsomal protein as the liver in the rat. A possible exception to this generalization is the intestine. The presence of the gut flora gives this organ considerable hydrolytic and reductive capability, which plays a major role in enterohepatic circulation. This is especially true in the rat, as it has a very effective biliary excretory capacity. The organs most frequently examined are the lung, liver, kidney, adrenal, and GI tract. While the metabolic capabilities of these organs may not necessarily play a major role in xenobiotic disposition, they can play major roles in target organ toxicity. This short review will highlight some examples of this phenomenon in the rat.

Adrenal Cortex

The adrenal cortex is a steroidogenic organ and, therefore, actually quite rich in cytochrome P_{450}–dependent MFO. (The testis and ovary are also quite rich in these enzymes, but will not be discussed here.) There are two different systems: microsomal and mitochondrial. The latter system is distinctive because of the presence of an additional protein, adrenodoxin, in the MFO system, which serves as the electron carrier between NADPH–cytochrome P_{450} reductase and the hemoprotein. These systems are primarily involved in the production of glucocorticoid and mineralocorticoid by this organ. As a generality, the toxicity associated with the MFO in the adrenal gland is not the

production of toxic reactive intermediates but is secondary to the effects of xenobiotics on adrenal hormone production. For example, 7a-thiosteroids, such as spironolactone, are suicide substrates and cause the destruction of adrenal cytochrome P_{450} (Menard et al., 1979; Sherry et al., 1988). Ketoconazole also blocks adrenal steroidogenesis by inhibiting cytochrome P_{450}–dependent activities (Loose et al., 1983). Veltman and Maines (1986a) reported that relatively small doses of mercury can cause major alterations in adrenal MFO activity. A single dose of $HgCl_2$ (30 nmol/kg sc) in male rats caused (24 hours) increases in adrenal mitochondrial cytochrome P_{450} accompanied by increases in steroid hydroxylase and side-chain cleavage activities. In contrast, adrenal microsomal cytochrome P_{450} was decreased, accompanied by decreases in 2lα-steroid hydroxylase activity. These effects would result in decreased corticosterone production and increased progesterone production. In a continuation on their investigations on heavy metals and adrenal hormone formation, Veltman and Maines (1986b) also reported that 7 days of treatment with cupric chloride significantly decreased O-hydroxylase activity, which resulted in a decreased plasma level of corticosterone. The metabolic concerns associated with TCDD are normally focused on the liver, but Mebus and Piper (1986) have demonstrated that TCDD can cause decreases in adrenal 2lα-hydroxylase activity and Brownie et al. (1988) reported that methylandrostenediol treatment decreased hydroxylase activity and that, therefore, the resulting accumulation of deoxycorticosterone may play a major role in androgen-induced hypertension in rats.

Hence, there are numerous examples of xenobiotics exerting potential toxic effects by disrupting adrenal steroid hormone production in the rat. It is important to note that such effects are not always accompanied by morphological evidence of adrenal damage. Unless one specifically examines for effects on adrenal steroidogenesis, these inhibitory effects on adrenal MFO are difficult to assess in the context of a routine toxicity study.

Gastrointestinal Tract

The GI tract has two separate identifiable sources of xenobiotic-metabolizing capability: that associated with the gut flora and that associated with the mucosa. As reviewed by Rowland (1988), the reactions catalyzed by the gut bacteria are largely hydrolytic and reductive in nature. In comparison to other species, rats tend to have the highest activity in glucuronidase, but also have high nitrate, nitrite, and azo-reductase activities. The high glucuronidase activity doubtlessly contributes to the high tendency of rats toward the enterohepatic circulation of xenobiotic metabolites. The metabolism of metronidazole by the rat provides an example of the important role that the gut flora can play in the metabolism of a synthetic chemical. N-(2-hydroxyethyl) oxamic acid is formed from metronidazole via the reductive action of intestinal bacteria. When metronidazole is given to routinely maintained laboratory rats, N-(2-hydroxyethyl) oxamic acid is excreted in the urine, whereas none is detected in the urine of germ-free rats (Koch and Goldman, 1979; Yeung et al., 1983).

The GI mucosa itself has low but detectable cytochrome P_{450}–dependent MFO activity in the rat. In humans, concentrations of the CYP3A family, the dominant drug-metabolizing P_{450} enzyme family, are 58 pmol/mg of CYP3A4 and 16 pmol/mg of CYP3A5 (Paine et al., 2006). Rat intestinal content of CYP3A enzymes is qualitatively and quantitatively very different (enzyme content in pmol/mg): Cyp3A62, 2.31; Cyp3a1, <0.02; Cyp3A2, <0.02; Cyp3A9, 0.78; Cyp3A18, 0.84 (Matsubara et al., 2004). There has been difficulty in evaluating species differences in the expression levels of UGT proteins because there have been few reports of selective antibodies available for each UGT (Komura and Iwaki, 2011; Miners et al., 2010). Even so, the relationship between intestinal MFO activity and chemical carcinogenesis has attracted considerable attention. Strobel and coworkers (1980), Oshinsky and Strobel (1987), and Tamura et al. (1987) reported that isolated gut mucosal microsomes are capable of the metabolism of many of the same model substrates (e.g., ethylmorphine, p-nitroanisole) as the liver, including the carcinogen benzo(a)pyrene. The system was induced by both β-NF and PB and inhibited by both SKF 525A

and 7,8 benzoflavone. The system produced positive results against known mutagens, which required metabolic activation in the Ames assay. In fact, MFO activity has been found along the entire intestinal tract in the rat; Bonkovosky et al. (1985) and Lindeskog et al. (1986) characterized small but measurable amounts of PB-inducible cytochrome P_{450} in the small intestine. Pascoe and Correia (1985) demonstrated that intestinal cytochrome P_{450} is regulated by both dietary selenium and iron concentrations; deprivation of either or both can lead to decreases in cytochrome P_{450}. Hence, it is clear that the GI tract of the rat possesses the ability to activate chemical carcinogens. Intestinal MFO can be manipulated in the same manner (with regard to inducers and inhibitors) as the liver MFO to study the activation of suspected GI carcinogens in vivo or in vitro.

Kidney

Litterst et al. (1975) were among the first to publish on the MFO of the kidney. On a microsomal protein basis, the rat kidney has about 13% of the cytochrome P_{450} as the liver, but only about 6% of the activity with aminopyrine, and scantly detectable activity with aniline. Endou (1983) examined the distribution of cytochrome P_{450} along the nephron in rats and found that it was localized only in the proximal tubule. Within the proximal tubule, the straight segment possesses higher amounts than the convoluted tubules. This was confirmed by Sugita et al. (1988); they also demonstrated that starvation induced cytochrome P_{450} in the convoluted tubule, whereas 3-MC induced preferentially in the straight portion. Cojocel et al. (1988) demonstrated that cephalexin rather specifically causes the depletion of cytochrome P_{450} in the rat kidney cortex. Babany et al. (1985) and Barry et al. (1987) explored the relationship between renal and hepatic MFO activity and noted that procedures (e.g., inhibition of the hepatic MFO, partial hepatectomy, cholestasis, and biliary cirrhosis) that decrease MFO activity resulted in increased renal MFO. These reports suggest that there is an inverse relationship between activities of the hepatic and renal MFO.

In an interesting similarity to the liver, the kidney also develops preneoplastic lesions that have different enzyme concentrations and staining characteristics than the surrounding normal tissue. Tsuda et al. (1987) demonstrated that the preneoplastic lesions induced by N-ethyl-N-hydroxyethylnitrosamine in the kidney and liver differed in that in the liver these foci contained decreases in all forms of cytochrome P_{450} examined and increased in epoxide hydratase, whereas in the kidney the altered foci contained increased levels of the cytochrome P_{450} isozyme PB3a and decreases in epoxide hydratase. This observation also demonstrates that the rat kidney also has considerable EH activity. Nephrotoxic doses of $HgCl_2$ induce large increases in renal EH activity. Greater increases were observed in Sprague Dawley than in Fischer 344 rats. Whether this indicates a role for EH in $HgCl_2$ nephrotoxicity, or whether this is a generalized response to nephrotoxicity, remains to be established.

Not only is the renal cytochrome P_{450} localized in the proximal tubule, but also anatomically located in the renal cortex of the rat and probably other species as well. Thus, agents that require metabolic activation via the MFO tend to produce renal damage that is restricted to the renal cortex. Acetaminophen, for example, causes renal cortical necrosis in rats (Newton et al., 1985). In addition, Fischer 344 rats are more susceptible, whereas Sprague Dawley rats are resistant. Newton et al. (1985) reported that when rats were treated with radiolabeled acetaminophen, the extent of covalently bound radioactivity in the renal cortex was much higher in Fischer 344 than in Sprague Dawley rats. These results clearly suggest that acetaminophen nephrotoxicity in the rat is due to metabolic activation and that strain-related differences are due to strain-related differences in renal MFO activity. Beierschmitt et al. (1986) reported that there were also age-related differences in the sensitivity of Fischer 344 rats to acetaminophen toxicity. At 600 mg/kg (ip),

acetaminophen caused severe renal lesions in old (22–25 months of age) rats, but no evidence of damage in 2- to 4-month-old rats and only intermediate damage in middle-aged (12–15 months) rats. Tarloff et al. (1988) confirmed this and further demonstrated that the aforementioned strain-related differences between Sprague Dawley and Fischer 344 rats became less noticeable as the animals aged. At 12 months of age, rats of both strains have equivalent sensitivity to acetaminophen nephrotoxicity, a finding not necessarily due to age-related differences in renal MFO activity.

Lungs

Relative to rat liver, the rat lung has even less MFO activity than the kidneys. According to Litterst et al. (1975), the lung has only about 7% of the cytochrome P_{450} as the liver (on a microsomal protein basis) and also only 35% of the microsomal protein. As reviewed by Mitchell and Boyd (1983), however, there is ample evidence that the lung has sufficient MFO activity to generate toxic reactive intermediate. In fact, for a few chemicals, such as 4-ipomeanol, the enzyme kinetic constants are such that MFO metabolism in the lung is favored over metabolism in the liver, so that toxicity is restricted to the lung (Mitchell and Boyd, 1983). Garst et al. (1985) demonstrated a positive correlation between pulmonary cytochrome P_{450} content and species differences in the pulmonary toxicity of 4-ipomeanol; the rat tends to be among the most sensitive of species.

Also as reviewed by Mitchell and Boyd (1983) and Guengerich (1990), the lung is extremely heterogeneous with regard to cell type. The Clara cells tend to be relatively rich, whereas type I epithelial cells are devoid of MFO activity. Keith et al. (1987) have demonstrated the presence of specific cytochrome P_{450} isozymes in both Clara and type 11 epithelial cells. Hence, toxins that required activation tend to have these two cell types as targets. The molecular biology and enzymology of pulmonary cytochrome P_{450} have been most thoroughly explored in the rabbit, but some work has been completed in the rat (Guengerich, 1990). Rat pulmonary cytochrome P_{450} exists as several different isozymes (some of which are indistinguishable from the corresponding isozymes of the liver). Pulmonary cytochrome P_{450} in the rat is induced by chemicals such as PB and 3-MC, and different isozymes are induced by different classes of inducers. For example, Robinson et al. (1986) reported that 3-MC induction increases the levels of a pulmonary cytochrome P_{450} that is identical to 3-MC-induced hepatic cytochrome P_{450}. Rampersaud and Walz (1986) demonstrated that rat lung contains at least six different cytochrome P_{450}s, one of which is cytochrome P-450b, whereas cytochrome P-450e (which is coinduced in the liver with cytochrome P-450b) was not induced under any circumstances in the lung. Hence, despite the similarities, the genetic controls over pulmonary cytochrome P_{450} differ somewhat from those of the liver.

In a fashion similar to the liver, however, different isozymes show different sensitivities to different inhibitors. For example, Rabovsky and Judy (1989) reported that rat pulmonary MFO activity toward benzyloxyphenoxazone was exquisitely sensitive in vitro to 1-octanol inhibition (IC50 ~ −3.8 pM), whereas activity toward ethoxyphenoxazone was completely unaffected by 1-octanol (at the limits of solubility). Naslund and Halpert (1984) reported that chloramphenicol (100 mg/kg ip or iv) is a selective suicide substrate for the pulmonary cytochrome P_{450} isozyme involved in the 2-hydroxylation of n-hexane. Elovaara et al. (1987) reported that inhalation of m-xylene (300 ppm) resulted in the selective destruction of pulmonary cytochrome P_{450} in rats with no effects on any other enzyme system and visible morphological changes. Pyykko et al. (1987) reported that various substituted benzenes also caused decreases in pulmonary cytochrome P_{450} and 7-ethoxycoumarin deethylation activity, but increases in 7-ethoxyresorufin deethylation activity. Rietjens et al. (1988) reported that prolonged (7 days) exposure of rats to ozone (by inhalation) results in increases in cytochrome P_{450} owing to proliferation of Clara cells. There was, however, a shift in cytochrome P_{450} isozymes such that activity toward

7-ethoxycoumarin decreases, whereas activity toward 7-pentoxyresorufin increased. Thus, the MFO of the rat lung is localized to two main cell types and (like that of the liver) is controlled by different cytochrome P_{450} isozymes. These different isozymes can be induced or inhibited by a variety of different chemical treatments.

Most of this discussion has focused on the pulmonary MFO. The rat lung, however, also has appreciable FMO activity (Tynes and Hodgson, 1983; Ziegler, 1988). This system has been shown to play an important role in the species selectivity of different rodenticides. For example, α-naphthylthiourea (ANTU) causes pulmonary edema in the rat, but not in larger species. Evidently, this difference is due to species differences in activity toward ANTU (and the formation of reactive metabolites), as reviewed by Mitchell and Boyd (1983).

Xenobiotic metabolism by the lung has important implications in toxicology, and (based on the preceding paragraph) the rat is an appropriate model for the study of this system. The first concern has to do with localized (pulmonary) toxicity due to the formation of reactive metabolites. Inhalation can result in direct exposure of the lung to relatively high (enzyme-saturating) amounts of potential toxins (although inhalation is not required for a chemical to be a pulmonary toxin, e.g., 4-ipomeanol). For example, 2-nitrofluorene is a potent carcinogen that is rapidly converted in vitro to the potent mutagen (and presumed ultimate carcinogen) 9-hydroxy-2-nitrofluorene by rat lung microsomes (Tomquist et al., 1988). Agents that induce pulmonary MFO activity will result in increased pulmonary sensitivity to procarcinogens. The second concern with regard to pulmonary MFO activity has to do with pulmonary first-pass metabolism of chemicals administered by inhalation. Metabolism by the lung can influence toxicity at more distal sites. Hexane, for example, is metabolized to a neurotoxic chemical, 2,5-hexanediol. Toftgard et al. (1986) reported that the lungs can facilitate the formation of this metabolite by catalyzing the formation of 2-hexanol, which is then transported to the liver for further metabolism to 2,5-hexanediol. These implications are of concern because for the majority of the general human population, inhalation is one of the major routes of incidental exposure to environmental and occupational chemical hazards. Thus, chemicals that influence pulmonary MFO activity may have potentially serious human health implications. As noted, pulmonary MFO activity can be altered under circumstances where no other noticeable effects occur, and this is the type of effect that would be easily missed in a traditional toxicology study. The rat would provide a good model for the study of the toxicological implications of pulmonary MFO induction or inhibition in a tier 2 or single endpoint type of study.

Transporters

When the author was a very young scientist in the 1980s, he had a PK data set that suggested that the molecule he was working on, a derivative of erythromycin, was being pumped back into the lumen of the intestine "after" absorption. The primary evidence was from a model using an isolated rabbit jejunum that had been inverted. The results showed that the compound could move against a 3–5× concentration gradient. Since the molecule was a xenobiotic and not endogenous, management dismissed the idea and suggested my results came from methodological errors. I accepted their decision and my acceptance is the greatest regret of my professional life. In recent years, it has become increasingly clear that drug transporters play an important role in the absorption, distribution, and elimination of drugs (Chu et al., 2013). These enzyme systems, much like the P_{450} enzyme systems, are also prone to induction and inhibition, which can be a mechanism for PK-based drug–drug interactions (DDIs). The current draft FDA Guidance on DDIs (FDA, 2012) goes into great detail about the agency's understanding of the importance of these formally little regarded enzyme systems. The following table outlines the transporter enzymes found in three cell types (Table 3.41).

To date, most identified transporters belong to one of two superfamilies: ATP-binding cassette (ABC) and solute carrier (SLC). Systems identified in the rat are listed in Tables 3.42 and 3.43.

Table 3.41   Outline of Clinical Examples of Efflux and Uptake Transporters in the Gut Wall, Liver, and Kidneys That May Be Involved in a Drug's Absorption, Distribution, Metabolism, and Excretion

Enterocyte

    Uptake (gut lumen to cell): PEPT1, OATP

    Efflux (cell back into gut lumen): MDR1, MRP2, BCRP

    Efflux (from cell to portal blood): MRP3

    Efflux or influx (from or into cell and portal blood): OCT

Hepatocyte

    Uptake (into cell from portal blood): OATP1B1, OATP1B3, OATP2B1, OCT1, OAT2

    Efflux (from cell into bile): MDR1, MRP2, BCRP, BSEP

    Efflux (from cell into systemic circulation): MRP3, MRP4

Renal tubule cell

    Uptake (from systemic circulation into cell): OCT2, OAT1, OAT3

    Efflux (from cell to renal lumen): MDR1, MRP2, MRP4, OCTN1, OCTN2, MATE1, MATE2

Source: FDA Guidance for Industry (2012) Drug Interaction Studies—Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations. U.S. Department of Health and Human Services, Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER) Draft, February.

Abbreviations: MRP, multidrug resistance–associated protein; PEPT, peptide transporter; OATP, organic anion transporting polypeptide; OAT, organic anion transporter; OCT, organic cation transporter; BCRP, breast cancer resistance protein; MDR, multidrug resistance (MDR1 is also known as P-glycoprotein or P-gp).

Table 3.42   Genetic Profiles of ABC Transporter Systems in the Rat

Gene Name	Aliases	Summary
Abca2	Abc2	ABC, subfamily A (ABC1), member 2; glycosylated ABC transporter that binds ATP in the presence of magnesium; may play an important role in brain function.
Abcb1b	Mdr1; Pgy1; Abcb1	ABC, subfamily B (MDR/TAP), member 1B.
Abcb1a	Abcb1; Mdr1a	ABC, subfamily B (MDR/TAP), member 1A; ABC multiple drug transporter P-glycoprotein that is activated during liver regeneration and hepatocarcinogenesis.
Abcb4	Mdr2; Pgy3	ABC, subfamily B (MDR/TAP), member 4; energy-dependent efflux pump; may be involved in changes in bile formation in response to diabetes.
Abcc1	Mrp; Mrp1; Abcc1a; Avcc1a	ABC, subfamily C (CFTR/MRP), member 1; ABC multiple drug resistance protein (MRP) involved in the release of GSH synthase during oxidative stress.
Abcc2	Mrp2; Cmoat	ABC, subfamily C (CFTR/MRP), member 2; multispecific organic anion transporter; mutation may be responsible for conjugated hyperbilirubinemia in the TR- rat, which is a model for human Dubin–Johnson syndrome.
Abcc3	Mlp2; Mrp3	ABC, subfamily C (CFTR/MRP), member 3; human homolog transports conjugated metabolites from hepatocytes into the bloodstream; may play a role in steroid metabolism.
Abcc4	Mrp4	ABC, subfamily C (CFTR/MRP), member 4; (ABC) MRP that mediates reduced GSH release from hepatocytes into blood by cotransport with monoanionic bile salts.
Abcc5	Mrp5; Abcc5a	ABC, subfamily C (CFTR/MRP), member 5; (ABC) MRP that mediates efflux cGMP during nitric oxide–induced regulation of smooth muscle.
Abcc6	Mrp6	ABC, subfamily C (CFTR/MRP), member 6; human homolog acts as a Mg-ATP-dependent efflux pump that transports GSH S-conjugates and mediates a low level of resistance to some anticancer agents.
Abcg2	BCRP1	ABC, subfamily G (WHITE), member 2.

Source: PubMed Gene database, http://www.ncbi.nlm.nih.gov/genbank/, accessed June 11, 2015.

Table 3.43   Genetic Profiles of SLC Family Transporter Systems in the Rat

Slc15a1	PEPT1	SLC family 15 (oligopeptide transporter), member 1, acts as a proton-dependent peptide transporter; may transport oral beta-lactam antibiotics across intestinal brush borders
Slc15a2	PEPT2	SLC family 15 (H+/peptide transporter), member 2, acts as a high-affinity proton-dependent peptide transporter; may transport peptides from cerebrospinal fluid to blood
Slc22a1	Oct1; Orct1; Roct1	SLC family 22 (organic cation transporter), member 1, polyspecific transporter involved in exporting ATP-dependent multidrug proteins through the basolateral membrane of renal proximal tubules
Slc22a2	OCT2; OCT2r; rOCT2	SLC family 22 (organic cation transporter), member 2, acts as a transporter for organic cations and nonneuronal monoamines; may play a role in organic cation/H+ antiport function in renal brush border membrane vesicles
Slc22a6	Oat1; Paht; Roat1; Orctl1	SLC family 22 (organic anion transporter), member 6, multispecific organic anion transporter; involved in anion transport in a proximal tubule in the kidney
Slc22a7	Oat2	SLC family 22 (organic anion transporter), member 7, may act as an organic ion transporter during liver development
Slc22a8	OCT3; Oat3; Roct	SLC family 22 (organic anion transporter), member 8, organic anion transporter; involved in homovanillic acid, an end metabolite of dopamine transport
Slc47a1	MATE1; RGD1311123	SLC family 47, member 1
Slco1a1	Oatp1; OATP-1; Slc21a1; Slc21a3	Mediates the transport of sulfated, amidated bile acid, sulfolithocholyltaurine, into rat hepatocytes
Slco1a2	Oatp2; Slc21a5; Slco1a4	Mediates transport of a wide range of organic anions including bile acids, estrogen conjugates, ouabain, and digoxin; may be involved in digoxin toxicity
Slco1a5	Oatp3; OATP-3; Slc21a7; Slco1a2	SLC organic anion transporter family, member 1a5pro, facilitates renal uptake and excretion of the uremic toxin indoxyl sulfate; may facilitate intestinal absorption of bile acids; transports thyroid hormones and taurocholate
Slco1a6	Oatp5; Slc21a13	SLC organic anion transporter family, member 1a6, may act as an organic anion transporter; expressed specifically in kidney
Slco1b3	Oatp4; OATP-4; rlst-1; Slco1b2; Slc21a10	SLC organic anion transporter family, member 1b3, mediated uptake of a variety of organic anions including taurocholate, bromosulfophthalein, and steroid conjugates
Slco4a1	OATP-E, Slc21a12	SLC organic anion transporter family, member 4a1, transports 3,3',5-triiodo-L-thyronine; may play a role in thyroid hormone metabolism

Source: PubMed Gene database, http://www.ncbi.nlm.nih.gov/genbank/, accessed June 11, 2015.

In contrast to metabolizing enzymes, which are largely concentrated in the liver and intestine, transporters are present with varying abundance in all tissues in the body. Transporters can also work in concert with metabolizing enzymes and play a role in drug metabolism.

As can be seen in the previous three tables, rats have many of the same types of enzyme systems as seen in man. As with drug metabolism, a drug's (or metabolite's) potential to affect the transport of other drugs should be evaluated. Use of *in vitro* tools to determine whether a drug is a substrate, inhibitor, or inducer of transport enzymes, followed by *in vivo* interaction studies to assess potential interactions, has become an integral part of drug development and regulatory review. Transporters can affect the safety profile of a drug by affecting the concentration of a drug or its metabolites in various tissues (FDA, 2012). Examples of clinically relevant DDIs

Table 3.44 Selected Transporter-Mediated Clinically Significant DDIs

Gene	Aliases	Tissue	Function	Interacting Drug	Affected Drug	ΔAUC[a]
ABC transporters of clinical importance in the absorption, disposition, and excretion of drugs						
ABCB1	P-gp, MDRI	Intestinal enterocyte, kidney proximal tubule, hepatocyte (canalicular), brain endothelia	Efflux	Dronedarone Quinidine Ranolazine Tipranavir/ Ritonavir	Digoxin Digoxin Digoxin Loperamide	2.6 fold 1.7 fold 1.6 fold 0.5 fold
ABCG2	BCRP	Intestinal enterocyte, hepatocyte (canalicular), kidney proximal tubule, brain endothelia, placenta, stem cells, mammary gland (lactating)	Efflux	CF 120918	Topotecan	2.4 fold
SLC transporters of clinical importance in the disposition and excretion of drugs						
SLC01B1	OATP1B1 OATP-C OATP2 LST-1	Hepatocyte (sinusoidal)	Uptake	Lopinavir/ ritonavir Cyclosporine Rifampin (single dose)	Bosentan Pravastatin Glyburide	5.48 fold 9.9 fold 2.3 fold
SLCO1B3	OATP1B3 OATP-8	Hepatocyte (sinusoidal)	Uptake	Cyclosporine Cyclosporine Lopinavir/ ritonavir	Rosuvastatin Pitavastatin Rosuvastatin	7.1 fold 4.6 fold 2.1 fold
SLC22A2	OCT2	Kidney proximal tubule	Uptake	Cimetidine Cimetidine Cimetidine	Dofetilide Pindolol Metformin	1.5 fold, 1.5 fold 1.4 fold
SLC22A6	OAT1	Kidney proximal tubule, placenta	Uptake	Probenecid Probenecid Probenecid	Cephradine Cidofovir Acyclovir	3.6 fold 1.5 fold 1.4 fold
SLC22A8	OAT3	Kidney proximal tubule, choroid plexus, brain endothelia	Uptake	Probenecid	Furosemide	2.9 fold

Source: Adapted from the FDA Guidance for Industry (2012) Drug Interaction Studies—Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations. U.S. Department of Health and Human Services, Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER) Draft, February.

[a] Change in the area under the plasma concentration–time curve (AUC).

due to transporter interactions are shown in Table 3.44. Therapeutic proteins and most other large-molecule agents (LMs) typically do not undergo metabolism or transport as their clearance pathway; therefore, the potential is limited for small-molecule drugs to affect LM metabolism or transport pathways.

The use of the rat as a model for DDIs is not mandated and is likely problematic. Even though the rat has many of the same enzyme systems as man, the local environment can be radically different. Care should be taken to ensure that the enzyme systems responsible for the clearance and distribution of the therapeutic agent under investigation in man, which can be determined *in vitro*, are adequately represented in the strain of rat used as a toxicological model.

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The Hamster

Shayne Cox Gad and Frederick G. Hess

CONTENTS

TOXICOLOGY

Shayne Cox Gad

The hamster is the third most frequently used laboratory animal after the rat and mouse at a level of ~146,000 per year (Renshaw et al., 1975; Silverman, 2012), though its use in toxicology is somewhat limited. While historically the hamster saw extensive use in carcinogenesis testing, as will be overviewed, this has changed. It has many attractive features as a laboratory animal because of its reproduction ease, unique anatomical and physical features, rapid physiological development, short life span, low incidence of spontaneous diseases, and a high susceptibility to induced pathological agents. Hamsters historically have been used in several fields, especially in carcinogenesis because of its low incidence of spontaneous tumors, but currently see most of their use in testing associated with buccal delivery of drugs (Gad, 2007). Hamsters have also played an important role in blood vessel physiology because their cheek pouches with thin vascularized walls are very accessible. The hamster is also a major model in diabetes research.

Taxonomy and History

Hamsters belong to the subfamily Cricetinae of the family Cricetidae in the order Rodentia. There are 50 species of hamsters in the Cricetinae, of which 8 species are maintained for medical research. Members of Cricetidae (also including the lemming, deer mice, and gerbils) are characterized by thick bodies, short legs, and large cheek pouches that are used to transport and store food. They have incisors that grow continuously and cuspidate molars that do not.

Species

The following is a discussion of the eight hamster species maintained in the laboratory. Table 4.1 lists these hamsters' common and scientific names and their chromosome numbers.

Syrian Hamster

The Syrian hamster is the most common laboratory hamster. Eighty percent of all hamsters used in research are Syrian. The remaining 20% are primarily Chinese, followed distantly by European,

Table 4.1 Common and Species Names and Chromosome Number

Common Name	Species Name	Chromosome Number
Syrian (golden)	*Mesocricetus auratus*	44
Chinese (striped, black)	*Cricetus griseus* or *barabensis*	22
European (common, black, field)	*Cricetus cricetus*	22
South African	*Mystromys albicaudatus*	32
Rumanian (newtoni's)	*Mesocricetus newtoni*	38
Turkish (kurdanti)	*Mesocricetus auratus*	42/44
Armenian (gray, migratory)	*Cricetulus migratorius*	22
Dzungarian (hairy-footed)	*Phodopus sungorus*	28

Armenian, Rumanian, Turkish, South African, and Dzungarian hamsters. The Syrian was originally native to the arid temperate regions of Southeast Europe and Asia Minor. It was first described as a new species (*Cricetus auratus*) in 1839. For almost 100 years, no hamsters were caught in the wild. The only proof that the species existed was the preservation in alcohol of two hamsters, one in London and the other in Beirut. Specimens of the species were finally obtained from the wild starting in the 1900s and have since been breed in multitudes in captivity. It lives in deep tunnels, which ensure cool temperatures and increased humidity. It is a nocturnal animal. The Syrian is virtually tailless and has smooth short hair. Normal coloration is reddish gold with a grayish white ventral portion. The dorsal side may have a black stripe. The ears are pointed with dark coloration, and the eyes are small, dark, and bright. The average life span is 2 years, but these animals can live up to 3 years. The animal is 14–19 cm in length and weighs 114–140 g at adulthood. The female is usually heavier and longer than the male.

The Syrian hamster was introduced into the laboratory in 1930 to study the Mediterranean disease kala-azar. Israel Aharoni (Hebrew University, Jerusalem, Israel) collected 11 young golden hamsters from Syria in 1930 while on a zoological expedition. The litter with their mother had been found in their burrow 2.5 m under a wheat field. Aharoni and his wife kept the hamsters in their house until one night when they all escaped. Nine hamsters were recovered and given to the animal facilities supervisor of the Weizmann & Seiff Institute, Jerusalem, Israel. Of the nine, five escaped the first night in the new facility, leaving only one female. The female was mated and gave birth to a litter of healthy pups. In a year's time, these hamsters produced more than 300 offspring and were the forbearers of today's laboratory-bred Syrian hamster. There is no record of any further captures of Syrian hamsters from the wild.

The Syrian has been involved in oncology, virology, endocrinology, physiology, parasitology, genetics, and pharmacology research. The cheek pouch of the Syrian hamster has provided the technology for studying microcirculation and the growth of human tumors.

Chinese Hamster

The Chinese hamster is native to China. It is 39–46 g in weight and 9 cm long at adulthood. It lives 2.5–3.0 years under laboratory conditions. Though the Chinese hamster is smaller than the Syrian, its testicles, spleen, and brain are larger.

In 1919, the Chinese hamster was used for the first time as an alternative to the mouse, which was extremely scarce at the time. The hamsters were used to determine the best therapy for the patients with pneumonia. The Chinese hamster was also used to study TB, influenza, diphtheria, and rabies.

Robert B. Watson, in December 1948 (right before the Communist takeover of China), was given 10 female and 10 male hamsters from C. H. Hu of the Peking Union Medical College. Watson placed the hamsters on what he believes was one of the last Pan Am flights out of China to San Francisco. From San Francisco, the hamsters were sent to New York. V. Schwenter of the Harvard Medical School obtained the hamsters and eventually successfully bred them in the laboratory. Of the original

20 hamsters, 4 of the females and 3 of the males produced offspring, which gave rise to the present Chinese hamster population. While the Harvard colony has since become extinct, colonies were established at the Upjohn Company (which became Pharmacia and most recently was acquired by Pfizer) in Kalamazoo, Michigan, and the C. H. Best Institute in Toronto, Ontario, in the 1960s.

The Chinese hamster has been used primarily in research for cytogenetics because of its low chromosome number (Fenner, 1986) and in diabetes mellitus because (a) some strains have very high incidences of the disease and (b) the course of the disease in this species is similar to that seen in humans.

European Hamster

The European hamster was first found in a West Germany industrial area. Its natural habitat is the lowlands of Central and Eastern Europe. The European hamster is a very aggressive animal, and in the wild, each adult lives in its own burrow. It has a white face and feet, and bodies are dorsally reddish brown and ventrally black with white patches laterally. They are about the size of a guinea pig, averaging 27–32 and 22–25 cm in length and weighing 450 and 350 g for males and females, respectively. Males reach sexual maturity at 60 days of age, whereas females at 80–90 days of age, and are mainly a seedeater. In the wild, they hibernate in the winter months. In their natural habitat, European hamsters can live up to 8 years, whereas under laboratory conditions, the average life span is 5 years. This reduction is believed to be due to the lack of hibernation afforded a laboratory-raised European hamster (Mohr and Ernst, 1987).

The European hamster has been used only in hibernation studies and in inhalation studies because its tidal volumes are the largest of any laboratory rodent species.

Armenian Hamster

The Armenian hamster is native to the Union of the Soviet Socialist Republic (USSR). Its body size, weight, care, and maintenance are similar to that of the Chinese hamster.

The Armenian hamster was first introduced as a laboratory animal in 1963. It was brought to the United States as a part of the USA–USSR Cultural Exchange Program. Scientists in the United States wanted to find more species of the dwarf hamster (like the Chinese), and the Armenian species has been the only species found. Although the Armenian hamster has been used on a limited basis, its research use has been in cytogenetics and oncology.

Turkish Hamster

The Turkish hamster is native to Iran and Turkey. It was originally trapped in 1962. As an adult, its average body weight is 150 g and its average life span is a little less than 2 years, though they have lived as long as 4 years. Some populations of the Turkish hamster have a diploid number of 42 and others have a number of 44. These hamsters interbreed readily and produce offspring with a diploid number of 44. Hamsters with a diploid number of 42 hibernate less than those with 44. Besides hibernation research, Turkish hamsters have been used in immunology, genetics, and reproductive behavior research (Cantrell and Padovan, 1987; Yerganian, 1972).

Rumanian Hamster

The Rumanian hamster was initially trapped and described in 1965. It is native to the Bucharest area and is used in the laboratories surrounding that area. Its care, size, and management are similar to that of the Syrian hamster, though it does not reproduce as well as the Syrian. The Rumanian hamster adult averages 100 g weight. Its face is more pointed and ratlike than the Syrian hamster, but it is similar in appearance to the Turkish hamster.

Dzungarian Hamster

The Dzungarian hamster is very timid. The males are 11 cm long and 40–50 g in weight; the females are 9 cm long and weigh 30 g at maturity. The Dzungarian hamster has a short tail about 1 cm in length, which is usually hidden by the body fur. The fur on the dorsal side is gray with a dark brown or black stripe from the nape of the neck to the base of the tail. The ventral fur is white. The average life span has been reported to be 1 year by Herberg et al. (1980) and 2 years by Heldmaier and Steleinlechner (1981).

The Dzungarian hamster, native to the USSR, was originally trapped in Siberia and provided to the United States by the USSR. The present Dzungarian hamster population is the result of the mating of one female to two males who were domesticated in 1965.

The Dzungarian has been used in research involving photoperiodism, the pineal gland, and thermoregulation.

South African Hamster

The South African is the only member of its genus and the only hamster native to Africa. The first colony was established in South Africa in 1941. In its natural habitat, South African hamsters are nocturnal, solitary burrowing rodents. Unlike other hamsters, it does not have cheek pouches. The hamster has gray-to-brown fur on its dorsal aspect with white on the ventral surface, feet, and tail. The tail is 5–8 cm long. Its ears are erect, and the eyes are dark and bright. Adult males and females weigh 145 and 95 g, respectively (Hall et al., 1967). The average life span is 2.4 years, with a maximum life span of 6.2 years (Davis, 1963). The United States received its first South African hamster in 1962. These hamsters have not had much of an impact in biomedical research except in diabetes mellitus and infectious disease research.

Husbandry

The reader is referred to Field and Sibold (1998) for a complete source in this area.

Housing, Caging, and Bedding

Hamsters should be housed singularly unless they have been housed together since weanlings. If raised together, hamsters will occasionally fight, though they tend not to cause each other too much physical harm (if of the same sex and if there are no offspring present). European hamsters should be housed one animal per cage and brought together only for breeding purposes.

Caging requirements are described in Table 4.2. Caging material is usually rigid plastic (polycarbonate, polystyrene, and polypropylene), galvanized metal, stainless steel, glass, and hard alloys of aluminum, but never wood (it will take a hamster only a short time to be free of a wooden cage, as they are great chewers). Cages may have solid or open wire mesh bottoms; however, hamsters tend to have fewer stress-related deaths and more rapid growth in solid-bottom cages with direct bedding.

If solid-bottom cages are used, materials such as straw, beet pulp, peat moss, hard wood chips, or wood (treated) shavings should be provided in the cage so that the hamster is able to build a nest to sleep in during the day. Straw may also be provided in cages with open wire mesh bottoms so that the hamsters may nest.

Pregnant females should not be housed in suspended cages with open wire mesh bottoms. They should be housed separately in solid-bottom cages with a caging material as described earlier. A nursing female and her young should have at least 121 in.^{2} of floor space. With dwarf hamsters, there should be at least 25 in.^{2} of floor space for the mother and her young.

Table 4.2   Space Recommendations and Regulations for Hamsters

Recommendations of the Laboratory Animal Resources National Research Council Guide for the Care and Use of Laboratory Animals (National Research Council, 1996)

Weight of Animal (g)	Type of Housing	Floor Area/Animal (cm²)	Height (cm²)
<60	Cage	64.5 (10 in.²)	15.24 (6.0 in.²)
60–80	Cage	83.9 (13.0 in.²)	15.24 (6.0 in.²)
81–100	Cage	103.2 (16.0 in.²)	15.24 (6.0 in.²)
>100	Cage	122.6 (19 in.²)	15.24 (6.0 in.²)

Regulations of Animal Welfare Act (PL89–544 as amended PL91–579) and the Animal Welfare Act, Code of Federal Regulations (1985).

Age	Minimum Space per Hamster (in²)		Maximum Population/ Enclosure
	Dwarf	Other	
Weanling to 5 weeks	5.0	10.3	20
5–10 Weeks	7.5	12.5	16
≥10 Weeks	9.0	15.0	13

Note: The interior height of the cage should be 5.5 in. for hamsters other than the dwarfs and 5 in. for the dwarfs.

Temperature, Humidity, and Lighting Requirements

Hamsters are generally more adversely affected by higher temperatures than lower ones. Temperature ranges for the nonbreeding hamster are 69°F–75°F (20°C–24°C) (Fox, 1979) and for breeding are 72°F–74°F. If temperatures fall below 4°C, the hamster will start to hibernate (Schermer, 1967).

Humidity requirements are 40%–60% with a lighting cycle of 12 hours light and 12 hours dark. This lighting also fulfills the requirement necessary for breeding. In uncontrolled light and temperature environments, failures in hamster reproduction have been observed in winter owing to decreased light and in the summer owing to increased temperatures.

Water and Diet

Water

Water should be available ad libitum. Water can be provided by an automatic watering system with a lixit accessible to the smallest hamster or by a water bottle with a sipper tube. Fluid requirements are 30 mL/day for the Syrian hamster, 11–13 mL/100 g/day for the Chinese hamster, and 5 mL/100 g/day for the European hamster.

Diet

The best diet for a hamster is one which is 16%–24% protein, 60%–65% carbohydrates, and 5%–7% fat. Most animal facilities use standard rodent chow for hamsters, though hamsters do have a higher requirement for zinc (0.6%), copper (10 ppm), and potassium (20 ppm) than rats (Newberne and McConnell, 1979). Copper may need to be increased during studies when the hamster may be extremely stressed and should be considered by the principal investigator. Some studies have shown that a soybean meal may offer better nutritional efficiency for hamsters. During lactation, nutritional requirements for female hamsters may increase, such as is seen in the rat. The female will show signs of extreme weight loss and maternal cannibalism during lactation if not enough nutrition is being provided.

Hamsters start eating solid food at days 7–10 of age. Syrian hamsters consume 5.5–7.0 g of food during their growth and development. Adult and pregnant hamsters usually consume 10–15 g/day.

Unlike rats, males and females consume nearly the same amounts of food. Hamsters, like other rodents, are coprophagic.

Feed is usually provided ad libitum. Hamsters do most of their eating at night, usually bingeing every 2 hours. Hamsters are hoarding animals and, therefore, will remove the food pellets from the feeder and pile them in a corner of the cage. The corner is usually opposite of the corner used for urination and defecation. Because of this trait, exact feed consumption for hamsters is difficult to determine. Feeders should have slots large enough to allow the hamster with its broad muzzle access to the food.

If hamsters are fasted for several hours or up to 4 days, they do not increase their food intake to compensate for the loss. If food is available only a certain time each day, a hamster will eat exactly as much as they would ordinarily eat if the food were ad libitum; however, hoarding activity is increased. This is in sharp contrast to the rat, which will compensate for periods of fasting.

Diseases and Spontaneous Tumors

Amyloidosis

Amyloidosis is a noninfectious disease, which occurs in aging hamsters. It is the principal cause of death of hamsters on long-term studies (Renshaw et al., 1975). In one report, a colony had an 88% incidence of the disease after 18 months of age. The onset of the disease may be due to a defect in the immune system because the first histopathological sign is deposition of immune globulins in blood vessels. Edema, proteinuria, hypercholesterolemia, and ascites have also been associated with the disease.

The incidence of amyloidosis varies from colony to colony. The amyloidosis is found in the liver, kidney, stomach, adrenal, thyroid, and spleen. The clinical signs and histopathological findings are similar to that seen in humans with the nephrotic syndrome.

Amyloidosis can be induced in adult Syrian hamsters by 1 mL sc injections (five times a week, once a day) of 50% casein Hammerstein in 0.3 M $NaHCO_3$, pH 7.5 (Gruys et al., 1979). Amyloidosis was induced in 8 weeks, first seen in the liver, spleen, and then the kidney. Amyloidosis can also be induced in animals treated with diethylstilbestrol (DES).

Antibiotic-Associated Enterocolitis

After treatment with gram-positive selective antibiotics, moribundity and mortality have been reported in hamsters. The cause of the disease may be due to the change in the intestinal microflora, which coupled with the small size of the animals may compromise their normal homeostasis (Bartlett et al., 1977).

Calcinosis

Calcinosis is characterized by mineralization of connective tissue in almost every organ of the hamster and is seen especially in the arteries. The early stage of the disease is characterized by the precipitation of mineralized material in the elastic fibers in the arteries. In the later stage, the lesions can spread to larger areas of the vessel.

Multifocal Retinal Dysplasia

Multifocal retinal dysplasia is found to have a 2% incidence in LAK:LVG Syrian hamsters. The disease can be detected from ophthalmoscopic examination in hamsters as young as 6 weeks of age and as old as 9 months. Ophthalmoscopically, the dysplasia foci are seen as either retinal streaks or small circular areas of cream-colored depigmentation. Histologically, the focal dysplasias are manifested as invaginations and rosette-like structures composed of elements of the photoreceptor layer, outer-limiting membrane, and outer nuclear layer (Schiavo, 1980).

Polycystic Disease

Hamsters have a cyst incidence of 76% at ages over 1 year (Gleiser et al., 1970). The liver is the most common site of the cysts (Renshaw et al., 1975). The lesions appear to be due to developmental defects of normal ductal structures such as the bile duct (Van Hoosier and Ladiges, 1984). No associated clinical signs have been reported.

Hamster Enteritis

Hamster enteritis (HE), or wet tail or wet tail disease, has also been called terminal ileitis, proliferative ileitis, and enzootic adenocarcinoma, among other terms. It is the most common and important disease of hamsters, especially the Syrian species. HE is a disease state in which the animals are excoriated, lethargic, irritable, anorexic, emaciated, and the caudal area is wet due to the diarrhea associated with the disease. After the onset of symptoms, death is usually 48 hours to a week later. The lesions seen are ulcerations of the cecal mucosa, inflammatory lesions of the ileum, cecum, jejunum, and colon, and the rectum contains yellow semifluid material. The specific cause is not known, but *Escherichia coli* have been associated with the etiology. It occurs at all ages, but it is seen especially just after weaning. The epizootiology and transmission is through direct contact and fomites. The best prevention and control of the disease is to obtain hamsters from suppliers with a minimal history of the disease. Orally administered antibiotics are the most commonly recommended treatment for this disease (Frisk, 1987).

Pneumonia

Next to wet tail, pneumonia is the most common disease affecting hamsters. The common causes are *Pasteurella pneumotropica*, *Streptococcus pneumoniae*, and *Streptococcus agalactiae*. The role of *Mycoplasma* sp. in pneumonia, though defined in mice and rats, has not been defined in hamsters. The clinical signs include anorexia, nasal and ocular discharge, and respiratory distress. Stress and significant variations in environmental conditions may be contributing and predisposing factors in the disease. Stressful situations should be avoided, and affected animals should be isolated. The diagnosis is made through the assessment of clinical signs, lesions, and microbiology laboratory results. If treatment is required, antibiotics to which the etiological agent is sensitive should be used.

Lymphadenitis

Lymphadenitis in the hamster is considered to be analogous to the condition seen in guinea pigs. In the hamster, the agent of etiology is either *Staphylococcus aureus*, β-hemolytic *Streptococcus* Lancefield group C, or *Streptobacillus moniliformis*. Cervical lymphadenitis is a chronic disease in which clinical signs appear several weeks after infection. Anorexia and death follow the observation of neck swelling owing to cervical lymph node abscesses (Owens and Young, 1973).

Tyzzer's Disease

Tyzzer's disease is seen more frequently in the mouse than in the hamster. The etiological agent is *Bacillus piliformis*. The clinical signs are diarrhea, dehydration, and lethargy. Lesions seen are enterocolitis, multiple white myocardial nodules, dilated cecum, colon containing semifluid stools, lymphadenitis, and multifocal necrotizing hepatitis (Zook et al., 1977). The diagnosis of the disease is by demonstration of *B. piliformis* in affected tissues following Giemsa or silver staining. Death is within 48 hours.

Salmonellosis

Salmonellosis is very rare in the hamster. The etiological organism is *Salmonella enteritidis*. The disease has a quick onset with death in a few days. Lesions seen are multifocal necrosis of the liver and septic thrombi involving the veins and venules. Affected animals, food, and bedding should be isolated to prevent further colony infections.

Lymphocytic Choriomeningitis

Lymphocytic choriomeningitis (LCM), which is transmittable to humans, is caused by a ribonucleic acid (RNA) virus of the arenavirus group. The clinical signs vary with the strain of the virus and of the hamster, the hamster's age, and the time of infection. Transmission is through direct contact, aerosol exposure, or fomites. Fifty percent of those infected as newborns or congenitally develop a chronic progressive fatal disease characterized by inactivity or weight loss (wasting disease). Findings at necropsy of those who develop the disease are chronic glomerulonephropathy and widespread vasculitis (Genovesi and Peters, 1987). To control for the disease, hamster colonies should be tested for antibodies, and infected animals eliminated. Hamsters implanted with tumors developed LCM and were shown to be antibody positive to the virus. The causative agent was the tumors. Students and staff working with the infected hamsters developed LCM, as did naive hamsters located near them. The spread of infection was halted by elimination of the infected hamsters and tumor cell lines (Biggar et al., 1977).

Sendai Virus Infection

Sendai virus infection is caused by parainfluenza 1 (Sendai). This virus is an RNA agent of the paramyxovirus group, which is related to the human virus hemadsorption type-2 (HA-2). Mice are believed to be the natural host, though rats, hamsters, and guinea pigs are susceptible to natural infections. Clinical signs are apparently asymptomatic. Others infected in vitro or as newborns or young adults develop subchronic infections. Consolidation of the lungs seen at necropsy has been reported in the hamster. In the absence of more reports, it is anticipated that gross and microscopic lesions would be similar to those seen in the mouse, i.e., an acute, interstitial pneumonia. The control and treatment of this virus in the hamster is the same as for the mouse.

Type C Virus Infection

Type C virus seems to be similar to the retrovirus type C oncovirus. The agent was first observed in human adenovirus–induced hamster tumors. One report indicates that lymphomas in hamsters may be associated with this agent. Transmission of type C virus is unclear, and it is believed that the agent may not be expressed until activation by chemical or physical agents or by infections caused by other oncoviral agents. In a study by Yabe et al. (1972), an untreated adult male hamster had an osteocarcinoma, which contained particles resembling type C virus. Yabe attempted to transmit the tumor with tissue extracts, but was not successful.

Parasitic Infections

Protozoa

Fecal smears of hamsters show the presence of a vast number and variety of protozoa. Protozoa may have a role in enteric diseases, though this is unclear because similar numbers and kinds of

Table 4.3   Location and Prevalence of Protozoa in the Hamster

Organism	Location	Prevalence (%)
Trichomonas sp.	Cecum, colon	99
Entamoeba muris	Cecum	33
Giardia sp.	Small intestine	9
Chilomastix bettencourti	Cecum	9
Hymenolepis nana	Small intestine	>1
Syphacia obvelata	Intestine	>1

protozoa are found in both healthy and sick animals. Table 4.3 lists the protozoa found most often in hamsters and the location and prevalence of these protozoa in a hamster colony (Wantland, 1955).

Nematodes

The major nematode infections in hamsters are caused by *Syphacia obvelata* (mouse pinworm) and *Syphacia muris* (ratoxyurid). The occurrence of *S. obvelata* is less than 1%; however, infection rates can be very high in individual colonies. The pinworm is found in the intestine, and the gross lesion is inflammation of the large intestine. Treatment is with 10 mg/mL piperazine citrate (in drinking water) twice in a 7-day period (Unay and Davis, 1980). *S. muris* is the consequence of direct contact with infected rats.

Mites

Acariasis in hamsters is caused by infestation by one of two species, *Demodex criceti* or *D. aurato.* Infection rates are high, though clinical signs of skin disease are very rare. Clinical signs seen included alopecia on the rump and back with dry, scaly skin. Ear mites in the hamster are caused by the *Notoedres* sp., tropical rat mite (*Ornithonysaus baacote*), and the nasal mite (*Spleorodens clethrionomys*).

Cestodes

Hymenolepis nana, the dwarf tape worm, and *H. diminuta* are found in the small intestine and are usually benign infections; however, when in large numbers, they may cause impactions and obstructions. Infection may also produce a mucoid or catarrhal inflammation of the bowel (enteritis). Diagnosis of the infection is by the observation of eggs in the feces or mature worms in the intestine. Newly acquired animals should be quarantined, and infected animals are isolated and treated with niclosamide.

Spontaneous Tumors

Table 4.4 lists the most common spontaneous tumors in hamsters by incidence and their incidence. The most frequent tumors are seen in the adrenal cortex and intestinal tract, followed by the lymphoreticular system, the endometrium, endocrine system, and ovaries of aging females (Barthold et al. 1978; Sher, 1982). The benign tumors found are usually adenomas of the adrenal cortex and polyps of the intestinal tract. In a study by Dontenwill et al. (1973), adenocarcinomas were age related with a rate of greater than 50% in hamsters over 100 weeks of age. The rate of small intestinal adenocarcinomas (0.8%) seen by Fabry (1985) was higher in hamsters than in rats or mice. Lymphosarcomas are the most common malignant tumors of the Syrian hamster. Tumors of the liver, pituitary, lung, urinary bladder, and mammary gland are practically unknown in the hamster, but these do occur

Table 4.4   Incidence of Spontaneous Tumors in Syrian Hamsters

Neoplasm	Males	Females	Total
Adrenal adenoma	12.7	9.4	11.0
Lymphoreticular neoplasm	3.7	2.3	3.0
Uterus endometrial polyp		3.0	
Uterus endometrial carcinoma		3.0	
Adrenal carcinoma	3.0	2.0	2.5
Pancreas islet cell adenoma	3.7	1.3	2.5
Vagina papilloma		2.0	
Stomach papilloma	1.7	1.7	1.7
Thyroid carcinoma	1.0	2.0	1.5
Uterus leiomyoma		1.0	
Small intestine adenocarcinoma	0.3	1.3	0.8
Pituitary adenoma	0.0	1.3	0.7
Pancreas islet cell carcinoma	0.7	0.7	0.7
Ovary fibroma		0.7	
Ovary theca cell tumor		0.7	

spontaneously in older rats and mice (Homburger and Bernfeld, 1979). Genetic drifts seen in many colonies of hamsters may influence the rate of spontaneous tumors as in the rat and mouse.

Animal Identification

Hamsters are usually identified by tagging, punching, or coding of the ear or ear tattooing, which is done aseptically.

Dosing Procedures

Oral Administration

To dose a hamster orally (p.o.), the animal is grasped by the skin of the neck and back. The gavage tube (metal 18 or 20-gauge) or a polyethylene catheter (2–3 cm in length) is passed into the mouth via the interdental space. The tube is passed gently into the esophagus and the fluid administered. The method is similar to the procedure done in the mouse and rat.

Subcutaneous Administration

The hamster is restrained for subcutaneous (sc) dosing as described for oral dosing. The needle is placed into the skin that is tented by pulling up a fold of skin on the back firmly between the thumb and index finger immediately behind the head. The injection is made into the skin parallel to the back. The hamster's loose skin enables large volumes to be injected sc in comparison to other rodent species of the same size (Collins, 1979).

Intradermal Administration

To administer an agent intradermally (id), the skin over the desired injection site is shaved. Holding the animal as described earlier, the needle (30-gauge) is advanced just a few millimeters into the skin. If there is suddenly no resistance, then the needle has been pushed through the skin. Withdraw and advance the needle again. Following administration of the material, a small welt will be visible.

Intramuscular Administration

To administer an intramuscular (im) dose, the muscles of the posterior and anterior thighs of the hamster are the most frequently used sites. The animal is restrained as described for oral dosing by an assistant, and one leg is held by the doser. The quadriceps is held between the forefinger and the thumb of the doser. The material is injected into the muscle mass.

Intraperitoneal Administration

To dose a hamster intraperitoneally (ip), the animal is grasped as described previously. The needle is pushed parallel to the line of the leg through the abdominal wall into the peritoneal cavity. Following the leg line avoids administration into the urinary bladder or the liver. Administration may occur when there is no resistance to the needle passage.

Intravenous Administration

It is best that the hamster is anesthetized to administer materials intravenously (iv). The veins that can be used are the femoral, jugular, and cephalic. The areas must be shaved, a skin incision made to expose the vein, and then a needle may be placed into the vein and the material administered.

Blood Collection Techniques

Retro-Orbital Method

The retro-orbital is the method of choice for collecting blood from the hamster. The method used is as described for the rat. A 23-gauge needle or a microhematocrit tube may be used to obtain the blood. Three milliliters of blood may be collected retro-orbitally; however, for repeated sampling, a volume of 0.5 mL is best for the animal. The use of anesthesia is preferable in the hamster.

Cardiac Puncture

Cardiac puncture in hamsters requires practice because the heart can be difficult to locate or can rotate away from the needle. A 25-gauge 3/a in. needle is the suggested equipment. A safe volume to draw from the heart with minimal damage is 1–2 mL. Repeated sampling from the heart is not advised because the mortality rate due to the blood withdrawn can be high (Wechsler, 1983). The use of anesthesia is suggested in the hamster. Upon exsanguination, 5 mL can be withdrawn from a 95 g hamster (Schermer, 1967).

Tail Clipping Method

The tail clipping method is good only for a maximum of six samples because the tail is so short. To facilitate blood flow, place a suction bell (which is connected to a water pump) on the base of the tail. Anesthesia is not necessary if the hamster is placed in a narrow tube with the hind legs protruding so they can be held.

Femoral Vein Method

To collect blood from the femoral vein, a tourniquet is placed above the stifle and the fur over the area clipped. A skin incision is made to expose the vein, and a 25-gauge 5/8 in. needle is placed into the vein. The blood is collected from the needle hub with a capillary microcontainer or a micro-hematocrit tube.

Jugular Vein Method

To collect blood from the jugular vein, the hamster should be anesthetized, the area shaved over the jugular, a skin incision made, and a 25-gauge 5/8 in. needle placed into the vein and the blood withdrawn.

Saphenous Vein

First, a body tube is used to immobilize the animal. The skin over the ankle is stretched, allowing for ease in shaving the area about the vein. A 25-gauge, 5/8 in. needle is slid into the vein, and blood is withdrawn. This method is currently the recommended approach to nonterminal blood collection (Hem et al., 1998).

Urine Collection

The best method is to collect the urine over 17–24 hours as the animal voids. A preservative such as thymol can be added to the collection vessel before starting. Catheterization of the ureter can be done; however, there is always a chance of blood or tissue contamination. A hamster's urine is normally a thick, milky fluid.

Analgesia and Anesthesia

Analgesia

Buprenorphine (0.5 mg/kg/sc) given every 8 hours provides good analgesia for the hamster. Hamsters are easily restrained if held by an experienced handler, and usually preanesthesia is not necessary; however, preanesthesia will be discussed.

Preanesthesia

Hypnorm (1 mL/kg/ip) provides sufficient analgesic for superficial procedures, and diazepam (5 mL/kg/ip) provides sedation, but no analgesia.

Anesthesia

Table 4.5 lists the concentrations of suggested anesthesias, site of administrations, and length of sedation (Flecknell, 1987).

Table 4.5   Anesthesia Data for Hamsters[a]

Compounds	Concentration	Time of Sedation
Fentanyl-fluanisone[b] + diazepam	1 mL/kg	60
Fentanyl-fluanisone-midazolam[b]	4 mL/kg	30–40
Ketamine-xylazine[b]	200: 10 mg/kg	70
Pentobarbital sodium	35 mg/kg	30–60
Thiopental sodium	40 mg/kg	
Alphaxolone-alphadolone[c]	15 mg/kg	20–60
Pentobarbital[c]	36 mg/kg	20–50

[a] Administered intraperitoneally.
[b] Provides surgical anesthesia.
[c] Provides sedation/light anesthesia.

Inhalation Anesthesia

To use inhalation anesthesia, the method is the same as described for the rat. The first choice for the hamster is methoxyflurane, followed by ether, halothane, and enflurane.

Euthanasia

The hamster may be euthanized by inhalation and by physical and parenteral methods. The best method is asphyxiation with carbon dioxide from dry ice or a gas cylinder followed by ether, halothane (respiratory arrest in 30 s), or methoxyflurane. The physical methods are cervical dislocation and decapitation with a guillotine (this method should be done only by an experienced researcher to prevent unnecessary pain and distress to the animal). Sodium pentobarbital (150 mg/kg) may be given *iv*, *ip*, intracardially (which may be painful), or into the thoracic cavity.

Physical Parameters

Neonatal Body Weights

At birth, Syrian hamsters weigh 2–3 g and Chinese hamsters weigh 1.5–2.5 g. Neonate hamsters are hairless with the ears and eyes closed. Table 4.6 lists information about the early development of the eight laboratory hamsters.

Body Weights and Weight Gains

The average adult body weight for each of the species is discussed in the species section of this chapter (Figure 4.1).

In a study by Borer et al. (1977), Syrian hamsters gained 2 g/day from birth to 5 weeks of age. From days 30 (weight = 65 g) to 70, the hamsters gained 1 g/day. From day 70 to 88, the hamsters gained 0.3 g/day. Syrian hamsters at maturity weighed from 100 to 135 g.

Normal fetal and maternal weight gain is well documented in Davis (1989).

Dentition

The dentition of a Syrian hamster is monophyodont, bunodont, and brachyodont. The incisors of the Syrian species grow irregularly depending on the age and sex of the hamster. The adult European hamster has one set of permanent teeth that consists of four continuously growing incisors and 12 molars.

Table 4.6   Neonatal Data for Hamsters

Species	Birth Weight (gm)	Day Eyes Open	Day Ears Open	Day Pups Solid Food	Day Weaned	Teeth at Birth
Syrian	2–3	15	5	7–10	21	Yes
Chinese	1.5–2.5	10–14	10–14		21–25	
Dzungarian	1.8	10	3–4	10	16–18	Yes
European						No[a]
Armenian		14	14	14	18	
Rumanian						
Turkish		12–13		12–13	20	
South African	6.5	16–25	3–5	21–25	No[a]	

[a] Incisors erupt at 3–5 days.
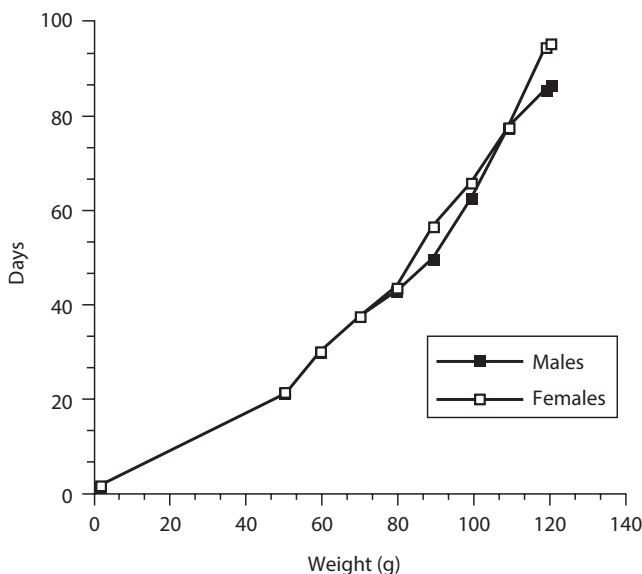
Figure 4.1   Charts the average weight of male and female Syrian hamsters over an average life span.

Life Spans

The average life spans for each of the laboratory species are discussed in the species section of this chapter.

Sexual Maturity

Sexual maturity for female hamsters begins at 4–6 weeks of age. At this time, there are 10 mature and 25 reserve follicles in each ovary. A female's first spontaneous estrus and ovulation are at 4–5 weeks and 30 days, respectively. In young immature females (4 weeks of age), ovulation may be artificially induced with 30 IU pregnant horse serum (Magalhaes, 1970). The estrus cycle is 94 hours or > 4 days in length with four distinct stages: proestrus, estrus, metestrus, and diestrus. Identification of the stage can be determined from differentiation of a vaginal smear through examination of cell types. During estrus (which occurs before and after ovulation), the female will show lordosis and will mate. The end of estrus is the appearance of a copious postovulatory discharge. The discharge is creamy, white, opaque, and very viscous with a pungent cheesy odor. Ovulation occurs regularly every 4 days, 9–10 hours after the peak concentrations of luteinizing hormone (LH). The breeding life of a hamster is usually about 10–12 months or after the production of six litters.

Syrian hamster males reach sexual maturity at 6–7 weeks of age and have a breeding life of about 1 year. The testicles of male Syrian and Chinese hamsters descend at days 26 and 30, respectively.

The secondary sex characteristic of the Syrian hamster is a scent organ (flank organ), which is located on the flank. The male touches the female's organ with his paws during copulation. The intensity of the pigmentation is an indication of androgen activity. The males have a darker pigmentation than females. The pigmentation is first seen at 25 days of age and is more marked at 35 days of age.

Breeding

Table 4.7 presents the basic parameters of hamster reproductive function.

Table 4.7 Reproductive Data for Hamsters

Species	Litter Size (Pups)	Gestation Period (Days)	Sexual Maturity	
			Males	Females
Syrian	11	16	6–7 weeks	4–6 weeks
Chinese	5	21	8–12 weeks	12 weeks
Dzungarian	4	18	35–40	90–139
European	7–9	15–17	60 days	80–90 days
Armenian	6–7	18–19		
Rumanian		16		
Turkish	6	14–15	7–8 weeks[a]	7–8 weeks
South African	3	38	4–7 months	4–7 months

[a] Occasionally do not reach maturity until 5–6 months of age, and then they may undergo spontaneous testicular regression.

Environmental Effects

With most laboratory hamster colonies maintained in controlled environments, seasonal changes in reproduction are rarely observed. In uncontrolled light and temperature environments, failures in hamster reproduction such as small litter sizes and lack of pregnancies have been seen in winter due to decreased light and in the summer due to increased temperatures.

Copulation

When a female in heat is placed with a male, there is a short period of investigation before mating. A female will demonstrate her willingness to mate by assuming lordosis, a posture where the back is held flat and firm with the legs braced and the tail is held erect and vertical. The male will groom the female and himself before mating. A male and female will repeatedly mate for 20–60 min with mounting and copulation taking place several times a minute. Rarely does a male attack a female during mating. An unreceptive female will attack a male and bite him on the face or scrotum. Females may also become aggressive after mating and will attack the male as described earlier.

Sperm penetration is usually 2–4 hours after ovulation. Fifty-six to three hundred and forty-three million sperm can be recovered from the female reproduction tract after copulation. This is approximately 40%–45% of the number of spermatozoa present in the vas deferens and epididymis of a mature male (Magalhaes, 1970). The observation of a copulation plug is an indication of successful mating. Examination for postovulatory discharge on days 5 and 9 of pregnancy will also confirm pregnancy. If a discharge is present, the female is having a normal estrus and therefore is not pregnant. If a discharge is present on day 10, the female is having a pseudopregnancy. In pseudopregnancy, the corpora lutea persist and function, but for a shorter time than a normal pregnancy. It is the result of copulation with a sterile male. Pseudopregnant females are excellent models to study decidual cell response and the formation of deciduas.

Pregnancy

If fertilization is successful (breeding day is day 0), on day 10, the female hamster shows an increase in body weight and a characteristic abdominal distension. Pregnancy length is 15 days and 7–17 hours. Gestation periods for Syrian hamster over 6 months of age and 1 year are over 16 and 17 days, respectively.

Parturition

Hours before delivery, the pregnant female becomes restless; alternates between eating, grooming, and nest building; and shows an increased respiratory rate. Hamsters that build small or tiny nests frequently have smaller litters or none at all. Active licking of the perineal region indicates the onset of birth. Young are born either breech or head first. They are licked clean and separated from the membranes and umbilical cord by their mother. Placentas are eaten immediately or stored as food. Litter sizes vary with the age of the female, genetic factors, types of diet, light, temperature, nesting material, and caging material. Litter sizes usually decrease in breeders older than 14 months. The male-to-female ratio at birth is almost equal.

During lactation, the mother shows excessive weight loss, which can be reduced if humidity is kept over 40% and animals have adequate water and food. Maternal cannibalism is common, particularly during the neonates' first week of life. The young appear to be biting the mother, and the mother retaliates by killing and eating the young. Other reasons for cannibalism include stress to the mother, especially if there are strange, loud noises, or if the litter is too large and the female reduces it to a manageable size. With Dzungarian hamsters, the mother and the young can be housed with the father. Both parents participate in the care of the offspring. The infants of a South African hamster remain attached to the mother's nipples and travel with her until they are 15–20 days of age. Since the female has only four nipples, the maximum litter size is four.

Respiratory Rate and Oxygen Consumption

The minimum respiratory rate (breaths/min) is 33, and the maximum is 127. The average respiratory rate is 74, and the mean resting respiratory rate is 30–33 breaths/min (Robinson, 1968). Hamsters are nose-breathers and have a resting oxygen consumption of 2.3 mL/g/h. It is possible to implant sensors to measure these, but such is uncommon in hamsters.

Body Temperature

Rectal temperature for Syrian hamsters is 99.5°F (37.5°C) and 36°C–37°C for the Dzungarian hamster.

Blood Pressure

The blood pressure measured by cannulation of the carotid artery of the hamster is 111 mmHg (Stroia et al., 1954). The blood pressure measured on the cheek pouch by Berman et al. (1955) was 90 ± 11.3 mmHg. Another measurement of blood pressure by photoelectric tensiometry was 108 mmHg. Though there cannot be a direct comparison of these measurements because of the techniques used, the values obtained are similar and can be used as reference values for each method described.

Heart Rate

The mean heart rate (beats/min) of the Syrian hamster is 450 with a range of 300–600.

ECG Patterns

The rate of contraction in the Syrian hamster is 350–500 beats/min. The P–Q interval is 48 ms with a range of 40–60, the Q–R–S interval is 15 ms with a range of 13–20, and the T- and P-wave amplitudes are 0.33–0.07 mV and 0.19–0.03 mV, respectively. For a measurement of ECGs, the hamster needs to be anesthetized because of its aggressive behavior. The ECG tracings of hamsters resemble human ECG tracings (Lossnitzer et al., 1977).

Clinical Laboratory

Clinical chemistry values for Syrian hamsters are listed in Table 4.8. The following will be a discussion of several interesting aspects of the hamster and its clinical chemistry values. In comparison to humans, the hamster has lower bilirubin, cholesterol, alkaline phosphatase, creative phosphokinase, lactic dehydrogenase (LDH), and A/G ratio values, and higher blood urea nitrogen, bicarbonate, phosphorus, amylase, acid phosphatase, aspartate aminotransferase (AST), and a2-globulin values. Since hamsters are such deep day sleepers, blood collection times should be noted because during light photoperiods, clinical chemistry values can be variable. The anesthesia used may also affect clinical chemistry values. These must be taken into account when analyzing clinical chemistry data.

Table 4.8   Clinical Chemistry Values for Syrian Hamsters

Test	Units	Male Mean Values	Female Mean Values	Range, Both Sexes
Bilirubin	mg/dL	0.42	0.36	0.20–0.74
Cholesterol	mg/dL	54.8	51.5	10.0–80.0
Creatinine	mg/dL	1.05	0.98	0.35–1.65
Glucose	mg/dL	73.4	65.0	32.6–118.0
Urea nitrogen	mg/dL	23.4	20.8	12.5–26.0
Uric acid	mg/dL	4.58	4.36	1.80–5.30
Sodium	mEq/L	128	134	106–146
Potassium	mEq/L	4.66	5.30	4.0–5.9
Chloride	mEq/L	96.7	93.8	85.7–112.0
Bicarbonate	mEq/L	37.3	39.1	32.7–44.1
Phosphorus	mg/dL	5.29	6.04	3.4–8.24
Calcium	mg/dL	9.52	10.4	7.4–12.0
Magnesium	mg/dL	2.54	2.20	1.9–3.5
Amylase	Somogyi units/dL	175	196	120–250
Enzymes				
Alkaline phosphatase	IU/L	17.5	15.4	3.2–30.5
Acid phosphatase	IU/L	7.45	6.90	3.9–10.4
Alanine transaminase	IU/L	26.9	20.6	11.6–35.9
Aspartate transaminase	IU/L	124	77.6	37.6–168
Creatinine phosphokinase	IU/L	101	85.0	50–190
Creatinine kinase	IU/L	23.1		
Lactic dehydrogenase	IU/L	115	110	56.0–170–0
Serum proteins				
Total protein	g/dL	6.94	7.25	4.3–7.7
Albumin	g/dL	3.23	3.50	2.63–4.10
α_1-Globulin	g/dL	0.64	0.55	0.30–0.95
α_2-Globulin	g/dL	1.85	1.70	0.9–2.70
β-Globulin	g/dL	0.56	0.83	0.1–1.35
γ-Globulin	g/dL	0.71	0.67	0.15–1.28
A/G ratio		0.87	0.93	0.58–1.24

Source: Mitruka, B. M. and Rawnsley, H. M., *Clinical Biochemical and Hematological Reference Values in Normal Experimental Animals and Humans*, 2nd ed., Masson, New York, 1981.

Glucose

Thiobarbiturate anesthesia may produce glucose levels as high as 300 mg/dL in adult male Syrian hamsters. The hyperglycemia can exist for up to 5 hours after anesthesia exposure, and there is no relationship to the length of hyperglycemia compared to the duration of the anesthesia. Hyperglycemia has also been reported in hibernating hamsters (Newcomer et al., 1987).

Lipids

The lipids found in the hamster are cholesterol, phospholipids, triglycerides, and fatty acids. Cholesterol concentrations in hamsters are the highest when compared to other laboratory animals, but lower than human levels. Short photoperiods (10 hours or less) can cause a decrease in cholesterol, but other lipids such as plasma triglycerides are not affected. Serum lipids do increase in the hamster during hibernation. A strain of spontaneous hypercholesterolemic Syrian hamsters shows increases in cholesterol when exposed to low temperatures.

Urea Nitrogen

Hamsters that develop kidney disease during the aging process have increased urea nitrogen levels as seen in other laboratory animals.

Enzymes

Serum obtained by cardiac puncture may be contaminated by AST, LDH, alanine aminotransferase (ALT), and creative phosphokinase owing to the high concentrations of these enzymes already present in the heart.

Alkaline Phosphatase

The alkaline phosphatase in the hamster is composed of isoenzymes from bone, liver, and intestine. Alkaline phosphatase is a more sensitive indicator of liver damage than bilirubin or ALT. Dramatic increases are usually indicative of bile duct obstruction. Immature hamsters have elevation of values two- to threefold compared to adults.

Alanine Aminotransferase

ALT is specific for liver damage in dogs, cats, and rats. In the hamster, ALT levels are increased in both acetaminophen-induced and viral-induced hepatic necrosis.

Aspartate Aminotransferase

The activity of AST is low but increases following muscle injury. Increased AST levels have been seen in hamsters with liver neoplasms.

Creative Kinase and Lactic Dehydrogenase

Cardiomyopathic Syrian hamsters show elevated creative kinase and LDH activities. Normal creative kinase levels are 23.1 IU/L, whereas in cardiomyopathic hamsters, the levels are 730 IU/L.

Thyroid Hormones

Thyroid hormones are of interest because of the hamster's hibernation. Basal T3 and T4 decrease with age. T_4 levels in 3-month-old hamsters are 6.75 ± 0.75 µg/dL and 3.59–0.16 µg/dL in 20-month-old hamsters. T3 levels in 3-month-old hamsters are 62 ± 2 ng/dL and 42 ± 3 ng/dL in 20-month-old hamsters. These changes are also seen in humans and in other rodent species. Older hamsters show less of an increase in T3 and T4 levels after thyroid-stimulating hormone (TSH) administration. During short photoperiods, there is a decrease in TSH, T3, and T4. Lower temperatures also cause decreases in T3 and T4 levels, whereas in cold conditions, there is an increase in T3 and a decrease in T4. Pregnant hamsters may metabolize thyroid hormones differently because there is a decrease in protein-bound iodine during pregnancy.

Reproductive Hormones

During estrus, there is one LH surge, and the follicle-stimulating hormone (FSH) is biphasic. The first burst of FSH occurs concurrently with LH. The second burst is thought to be responsible for the initiation and/or maintenance of follicular growth for the next estrus cycle. Maintenance of functional corpora lutea is believed to be performed by a combination of prolactin, FSH, and a small amount of LH.

Progesterone is the dominant hormone during the first 2 days of estrus, decreasing on day 3 but rising again on day 4. The levels of progesterone on days 1–2 are dependent on LH. Estradiol levels are low for the first 2 days and increase and decrease on day 4.

Adrenal Hormones

In the hamster, corticosterone and cortisol are secreted by the adrenal cortex. Corticosterone levels are three to four times higher than cortisol during the day. Adrenocorticotropic hormone stimulation increases both hormones; however, cortisol levels are stimulated at a higher rate. Basal cortisol levels are 0.45 ± 0.04 µg/dL and 0.38–0.09 µg/dL in males and females, respectively, and corticosteroid levels are 7.4–1.9 mg/dL (Tomson and Wardrop, 1987). Pregnant hamsters can produce large quantities of cortisol (30 µg/dL), whereas nonpregnant females have relatively low levels (0.3 µg/dL) in comparison to other species. Increased plasma cortisol levels are seen after exposure to chronic stress.

Glucocorticoid levels follow the circadian pattern seen in other rodents.

Proteins

Chinese hamsters with spontaneous diabetes have 10%–30% of their total proteins being α_2, whereas control hamsters have only 3%–8%. Asymptomatic hamsters with significantly elevated α_2 proteins do develop chemical or clinical diabetes later on.

Hematology Values

Hematological values for Syrian hamsters are listed in Table 4.9. Hematological values for European and Chinese hamsters are listed in Table 4.10. The blood volume of a hamster is 6%–9% of body weight. The maximum safe volume for one bleeding is 5.5 mL/kg. The practical volume from adult hamsters for diagnostic use is 1 mL. Hematological values for a hamster vary considerably because they are deep day sleepers, though values between males and females are similar. The variations seen are changes in blood volume and quantity of blood components.

Table 4.9   Hematological Values for Syrian Hamsters

Test	Units	Male Mean	Male Range	Female Mean	Female Range
RBC	×10E6/mm^3	7.5	4.7–10.3	6.96	3.96–9.96
HgB	g/dL	16.8	14.4–19.2	16.0	13.1–18.9
MCV	µ^3	70.0	64.0–77.6	70.0	64.0–76.0
MCH	Mµg	22.4	19.9–24.9	23.0	20.2–25.8
MCHC	%	32.0	27.5–36.5	32.6	27.8–37.4
Hct	%	52.5	47.9–57.1	49.0	39.2–58.8
Sedimentation rate	mm/h	0.64	0.32–0.96	0.50	0.30–0.70
Platelets	×10E6/mm^3	410	367–573	360	300–490
WBC	×10E6/mm^3	7.62	5.02–10.2	8.56	6.48–10.6
Neutrophils	×10E6/mm^3	1.68	1.11–2.25	2.48	1.88–3.08
Eosinophils	×10E6/mm^3	0.07	0.04–0.12	0.06	0.04–0.08
Basophils	×10E6/mm^3	0.08	0.05–0.10	0.04	0.03–0.05
Lymphocytes	×10E6/mm^3	5.6	3.69–7.51	5.81	4.41–7.20
Monocytes	×10E6/mm^3	0.19	0.12–0.26	0.20	0.16–0.25

Source: Mitruka, B. M. and Rawnsley, H. M., *Clinical Biochemical and Hematological Reference Values in Normal Experimental Animals and Humans*, 2nd ed., Masson, New York, 1981.

Table 4.10   Hematological Values for European and Chinese Hamsters

Parameter	Units	European Range	Chinese Range
RBC	×10E6/mm^3	6.04–9.10	4.4–9.1
HgB	g/dL	13.4–15.5	10.7–14.1
MCV	µ^3	58.7–71.4	53.6–65.2
MCH	µµg	18.6–22.5	15.5–19.1
MCHC	%	26.4–32.5	27.0–32.0
Hct	%	44.0–49.0	36.5–47.7
WBC	×10E6/mm^3	3.4–7.6	2.7–9.6
Neutrophils	×10E6/mm^3	3.5–41.6	14.8–23.6
Eosinophils	×10E6/mm^3	0–2.1	0.3–3.1
Basophils	×10E6/mm^3	0–0.2	0.0–0.5
Lymphocytes	×10E6/mm^3	50.0–95.0	68.1–84.8
Monocytes	×10E6/mm^3	0–1.0	0–2.4

Source: Mitruka, B. M. and Rawnsley, H. M., *Clinical Biochemical and Hematological Reference Values in Normal Experimental Animals and Humans*, 2nd ed., Masson, New York, 1981.

Erythrocytes

Erythrocytes in the hamster have a diameter of 5–7 µm. South African hamsters have larger erythrocytes than other hamsters and laboratory rodents. A small portion of erythrocytes show polychromasia (Schermer, 1967). Erythrocytes have a life span of 50–78 days. Desai (1986) saw an increase in erythrocyte longevity during hibernation. Nucleated or basophilic cells are rare, but reticulocytes are found from 3% to 4.9%.

Leukocytes

Hamster leukograms are similar to those of other laboratory rodents. During photoperiods, the total leukocyte counts range from 5,000 to 10,000/μL, whereas during hibernation, counts drop to 2,500/μL. During sleep, the lymphocyte: neutrophil ratio is 45%: 45%, whereas in awake animals, the neutrophil percentage varies between 17% and 35%.

Coagulation

Hamster blood starts to coagulate at 15–20 s (Schermer, 1967) with a mean coagulation time of 142 s (Desai, 1986).

Trypanosomes

Hamsters frequently have trypanosomes in the blood. These microorganisms are parasitic but not pathogenic. They have been observed in other laboratory animals, including sheep and monkeys, but not in the large numbers as seen in the hamster. The counts of trypanosomes can be very large, sometimes equaling the leukocyte counts. Professor Enigk of the Bernhard-Nocht Institute, Germany (personal communication) found that 50% of his hamster colony had trypanosomes. Transmission is not known; however, trypanosomes are not considered harmful and will not interfere with the outcome of a hamster study (Schermer, 1967).

Blood Gases and pH

The values for blood gases are Pa_{O_2} 71.8 ± 4.9 mmHg, Pa_{CO_2} 41.1 ± 2.4 mmHg, HCO_3 29.9 ± 2.9 mEq/L, and the blood pH is 7.48 ± 0.03. Measurements of exercising hamsters show an increase of 12.9 ± 7.9 in Pa_{O_2}, a decrease of 6.6 ± 2.6 Pa_{CO_2}, and a decrease of 3.5 ± 2.3 in HCO_3 concentrations.

Urine Values

The range of urine volume is 5.1–8.5 mL/24 hours in normal hamsters. In diabetic Chinese hamsters, the urine volume can be as high as 25 mL/day. Sodium and potassium concentrations are 70 and 120 mmol/L, respectively. The pH is basic, proteins are excreted about 10 times the rate of humans, and cholesterol is the main lipid excreted.

Species Peculiarities

The hamster cheek pouch is unique because it accepts heterologously neoplastic tissue but rejects normal human tissue. This led to the discovery that a biological difference existed between malignant and nonmalignant tissues. The cheek pouch has been used for the transplantation of neoplastic tissue for the evaluation of growth. When this method was standardized, it became a screening tool for chemotherapeutic agents (Newcomer et al., 1987).

The cheek pouch is transparent and very accessible. It is ideally suited for in vivo studies of microcirculation and the behavior of formed blood elements. These features have also made the hamster the model of choice for buccal administration and evaluation of oral and mucosal irritation. The hamster cheek pouch is, indeed, the standard model for evaluating acute or cumulative (28 day) irritation/tissue tolerance, as well as providing a popular model for the induction and study of squamous cell neoplasia (Heller et al., 1996).

Strain-Related Considerations

In a study by Althoff and Mohr (1973) comparing the chronic respiratory response of the Chinese, Syrian, and European hamsters to diethylnitrosamine (DEN) and dibutylnitrosamine (DBN), strain-related differences were found. In the Chinese hamster, DEN did not produce neoplasms in the respiratory tract. In the Syrian species, DEN caused tumors in the trachea and lungs followed by the nasal cavity. In European hamsters, DEN produced benign and malignant tumors in the respiratory tract and caused death after 15 weeks of daily treatment.

In the Chinese hamster, DBN caused papillary tumors and malignant neoplasms in the nasal and paranasal cavities. In the Syrian species, DBN caused tumors, primarily in the trachea, then the nasal cavities and lungs. At the high dosages in European hamsters, DBN caused carcinogenic effects in the trachea, lungs, and nasal cavities, whereas low-dosage groups had lung carcinomas.

As indicated by this study, hamsters are good models to study respiratory carcinogenesis; however, different species can have different responses to a chemical. The spontaneous rate of respiratory tumors, metabolism, and nature of chemical needs to be known before cross-species extrapolation can be done.

Typical Study Protocols

Carcinogenicity Toxicity Testing

Hamsters are highly suitable animals for carcinogenicity testing because of a low incidence of spontaneous tumor development, but they are highly susceptible to experimentally induced carcinogenesis. The incidence of spontaneous tumors in Syrian hamsters is reported to be lower than the incidence seen in mice or rats (Homburger et al., 1979; Mohr, 1979). Though the hamster does have a short life span, substance-related effects and neoplasms develop rapidly, during which spontaneous diseases and tumors may not occur. However, hamsters are infrequently used in testing, and a 2004 inquiry of (the more than 90) known contract laboratories succeeded in identifying only one with current experience in performing such studies.

The carcinogenicity protocols used for rats are satisfactory for hamster studies. Changes that need to be incorporated are that blood collections should be kept to a minimum and the length of the study usually is shortened (to 96 weeks) owing to the hamster's shorter life span.

Hamsters are specifically recommended for long-term testing with aromatic amines, polycyclic hydrocarbons, and other agents suspected of being pulmonary carcinogens (Aufderheide et al., 1989). Urinary bladder carcinomas induced by aromatic amines can take up to 7 years to induce in dogs, but can cause neoplasms in less than 1 year in hamsters (Witschi et al., 1993).

Nitrosamines caused tumors in the hamster forestomach, liver, pancreas, nasal cavity, lung, trachea, and occasionally the esophagus. The common site for nitrosamine tumor induction in the rat is the esophagus, demonstrating species specificity for a target organ site. Hamsters are not more susceptible to nitrosamines because some nitrosamines are more toxic in the rat than the hamster and vice versa (Newcomer et al., 1987). The hamster does show a nitrosamine-induced pancreatic tumor similar to pancreatic tumors in humans.

Inhalation and Intratracheal Studies

Inhalation studies constitute a significant portion of the toxicological research using the hamster as the test species. The hamster is deemed useful because it has a lower incidence of spontaneous respiratory tumors and of respiratory diseases (Werner et al., 1979), and its respiratory epithelium is more similar to that of the human than other laboratory rodents. The hamster has similar lung

absorption characteristics to those of the rat and mouse for aldehydes, ozones, and other irritant gases (Karube et al., 1991; Morris, 1997; Steinberg et al., 1990). Due to its more mixed breathing pattern, it compares favorably to the rat (an obligatory nose-breather) for studying fiber and particulate inhalation (Geiser et al., 1990; Gelzleichter et al., 1999; Hesterburg et al., 1997; Warheit and Hartsky, 1993; Warheit et al., 1997). In studies with cigarette smoke, certain inbred species of the hamster are the only laboratory rodents where carcinogenesis can be induced by inhalation. A laryngeal cancer in the hamster is caused by tar fractions or cigarette smoke. This cancer has been found to be histologically identical to the cancer seen in humans (Homburger et al., 1979). The species thus continues to be popular in studying the toxicity of cigarette smoke and its mechanisms (DiCarlantonin and Talbot, 1999; Tabassian et al., 1993).

Acute and subacute inhalation toxicity studies using the hamster have studied nickel monoxide (NiO), cobaltous oxide (CoO), and chrysotile asbestos. Hamsters exposed to asbestos for 11 months developed asbestoses and those exposed to NiO developed pneumoconiosis, occupational disease states seen in humans.

In whole-body exposure inhalation study designs, the exposure chamber has to be large enough to allow an adequate number of animals to be exposed simultaneously. It should be equipped with the means to regulate temperature and humidity and have identical chambers for all treatment groups. The hamsters should be housed separately; however, if there are space limitations, animals may be housed in groups. The position of the cages should be rotated from exposure to exposure. The animals should be housed in an area other than the inhalation chamber when not being treated. This will reduce the contamination of the exposure chamber by bodily fluids.

Current practice makes nose-only exposure with animals restrained in tables more popular, but hamsters do show extensive stress-related physiology responses to such restraint, including marked body weight loss (King-Herbert et al., 1997).

Animals are randomly distributed to test groups based on body weights as performed in other types of animal studies. The animals should have free access to water at all times. Feed should be available when animals are not being exposed unless exposure times are very lengthy. If feed is provided during exposure, then the feed is also being exposed to the test materials and may be an important aspect of the study. The number of exposures whether once or several times a day and the length of exposure time can be decided by the investigator; however, once decided, exposure time should remain consistent throughout the study. The concentration and particle size of the aerosol should be determined periodically, and the aerosols should be evenly distributed in the chamber (Raabe et al., 1973). Data may be collected concerning clinical signs, body weights, pharmacokinetics, mortality, hematological and clinical chemistry functions, organ weights, and gross and microscopic observations.

For intratracheal instillation studies, the same procedures as described for inhalation studies are used except animals are exposed to the control and test articles via intratracheal administration. The common dose volume is 0.2 mL per animal, and the animal is usually anesthetized during dose administration. The number of treatments per day and the length of the study may be decided by the principal investigator.

Teratology Studies

The hamster has been a popular alternative species for teratology and reproductive toxicity studies because of its predictable estrus, short pregnancy period, rapid embryonic development, and a low incidence of spontaneous malformations (DeSesso et al., 1998; Gomez et al., 1999; Williams et al., 1991; Wlodarezyk et al., 1995; Wolf et al., 1999).

Retinoic acid (vitamin A) has been shown to be a teratogen in hamsters (Eckholf and Willhite, 1997; Frierson et al., 1990; Willhite et al., 1996, 2000). Thalidomide was found to be a teratogen in certain inbred strains of the Syrian hamster making it a viable alternative to rabbits. Other hamster teratogens are dinocap (Rogers et al., 1989), cyclophosphamide (Shah et al., 1996), hydrocortisone,

colchicine, vincristine, vinblastine, heavy metals such as cadmium compounds, organic and inorganic mercury, 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), and 2,4-D (2,4-dichlorophenoxyacetic acid) alone or contaminated with dioxin (Newcomer et al., 1987). The study design for teratology studies should take into account the embryonic development of the hamster, the strain-specific fertility seen in hamsters, and the age of the mother.

Toxicology Studies

The protocols used for rats in acute and long-term toxicity studies are satisfactory for the hamster; however, blood collection should be kept to a minimum and the length of the test may need to be adjusted (shortened due) to the shorter life span of the hamster.

The majority of toxicology work involving hamsters has been buccal inhalation and respiratory studies, though intratracheal has also been a popular route due to the possibility of precise control of doses (Biswas et al., 1993). The hamster has been found to be a useful model in the study of toxicity; however, it has some biochemical and physiological characteristics not seen in other rodent species. The hamster has a strong resistance to certain pharmacological agents such as barbiturates, morphine, and colchicine, but is very sensitive to halothane- and sevoflurane-induced changes in diaphragmatic contractility (Kagawa et al., 1998). The hamster oral LD_{50} of colchicine is 600 times the lethal dose known to humans, whereas morphine when given to hamsters at the LD_{50} does not produce a narcotic reaction. These characteristics should not be considered a barrier to the use of hamsters in toxicology studies.

Of 304 compounds evaluated by the IARC, 130 were carcinogenic in at least one rodent species. Of the 130, only 38 compounds were tested in both hamsters and rats, 35 were tested in hamsters and mice, and 78 tested in both rats and mice. Of those tested in hamsters and rats, 84% of the compounds had similar results in both species, 86% in both mice and hamsters, and in mice and rats 90% had similar results. Based on this information, hamsters are not more or less sensitive to toxicity than other rodent species used in long-term testing (Arnold and Grice, 1979).

Chinese Hamster Ovary Cell Chromosome Aberrations

The purpose of this assay is to evaluate the ability of a compound to induce chromosome aberration clastogenic responses in the Chinese hamster ovary (CHO) cells. The realization that fully 60% of drugs listed in the *Physicians' Desk Reference* have positive CHC chromosome aberration findings associated with them has raised concerns about this assay.

The CHO cells used in this assay may be obtained from the American Tissue Culture Collection, Rockville, Maryland (Brusick, 1982). The original cells were obtained from a Chinese hamster.

The assay is divided into two parts, nonactivation and activation, with S9 rat liver as the activating agent. EMS (0.5 μL/mL) is the positive agent for the nonactivation, whereas for the activating studies, it is dimethylnitrosamine (DMN) at 0.5 μL/mL. The solvent used to dissolve the test article is used as the solvent vehicle for the control and positive control articles. The dosages selected for the test article are one toxic (loss of growth potential) and four lower (usually in a half-log series) concentrations. These dosages are determined in a range finder with the cells exposed to the test article for 4 hours and incubated for 24 hours.

The CHO cells are grown in 10% fetal calf serum/Ham's F12 media. The cell density should be kept at 1.5×10^6 per 75 cm^2 plastic flask. For the assay, approximately 0.25×106 cells per well per test article concentration are tested. The cells are exposed to the test article for 2 hours at 37°C. Cells used in the activation section receive the S9 rat liver along with the test article before the 2-hour activation. The cells are then washed with sterile saline and given fresh media. For each of the test article dosages, half of the plates per treatment group will receive 5-bromo-2′-deoxyuridine at a concentration of 10 μM. The cells are then incubated for 24 hours with colcemid (2×10^{-7} M)

added at hour 17. After incubation, the metaphase cells are collected by mitotic shake-off. These cells are swollen with 0.0075 M KCl solution, washed with a methanol: acetic acid (3:1) fixative, dropped onto glass slides, and air dried. The slides are stained with 10% Giemsa (pH 6.8), and the slides are scored for chromosomal aberrations such as chromatid and chromosome gaps, breaks, and chromatid deletions.

Syrian Hamster Embryo Cell Transformation Assay

Another genotoxicity assay currently being promoted for use as an alternative for the CHO and a screening assay for clastogens is the Syrian hamster embryo (SHE) (LeBoeuf et al., 1996). The SHE cell transformation assay evaluates the potential of chemicals to induce morphological transformation in karyotypically normal primary cells. Induction of transformation has been shown to correlate well with the carcinogenicity of many compounds in the rodent bioassay. Historically, the assay has not received widespread use due to technical difficulty (Leonard and Lauwerys, 1990). This was the subject of an entire issue of Mutation Research (1996) and was further evaluated by regulatory agencies, using a low-pH technique, which is technically easier to use and reproduce (Aadema et al., 1996; Isfort et al., 1996; Kerckaert et al., 1996; LeBoeuf et al., 1996; Custer et al., 2000). While the test still has advocates, it has not been widely adopted.

Models of Diseases

Cardiomyopathy

Cardiomyopathy in hamsters originates from a genetically (recessive autosomal gene) determined metabolic defect that induces degenerative lesions in all striated muscles with particular intensity and consistency in the heart (Bajusz, 1969; Gertz, 1973). The clinical and pathological aspects of the disease resemble nonvascular myocardial disease in man. Animals appear normal, though there is cardiac muscle degeneration. The lesions appear histologically in both sexes at 350 days of age for males and 25–30 days for females. The first lesion is an acute myolysis with primary dissolution of the myofilaments. This lesion is healed and replaced by connective tissue by day 100 (Gertz, 1973). The disease becomes clinically apparent with a whole-body sc edema; however, there are no ECG changes to foretell edema formation or to tell degree of lesion formation. The ECG changes are observed after lesion formation and consist of alterations in pathways of cardiac excitation and high-frequency alterations in the QRS interval. In the late stages of the disease, ascites, hydrothorax, and hydropericardium appear. In the terminal stage, animals are hyperpneic and cyanotic. At necropsy, the liver, spleen, kidney, heart, and other visceral organs show congestive changes such as enlargement and increases in volumes. Cardiomyopathic hamsters show an edema respond to therapy with digitalis, diuretics, and salt restrictions. The average life span of hamsters with this disease is 146 days. Since this model responds to therapy in a manner similar to humans and the disease state has similar manifestations, it is considered well suited to study heart failure in humans due to cardiac muscle impairment.

Dental Caries

Caries is a disease of poorly developed and poorly calcified teeth in the hamster. It is considered transmissible and infectious. It can be induced in normal hamsters by inoculation with cariogenic microflora. Animals are infected by adding microflora to drinking water or exposing the animals to infected feces. The carious lesions are in the molars. The lesions start with changes in enamel translucency, surface depressions, and fissures. The disintegration goes into the dentine with eventual exposure of the pulp. Bacterial (putrefactive) infections, inflammation, and complete necrosis

of the molar occur. With the testing of this model, fluoride was found to be beneficial to hamsters, and this prompted clinical trials in humans (Keyes, 1960).

Diabetes Mellitus

Diabetes mellitus was first described in 1959 and in 1969 in the Chinese hamster and South African hamsters, respectively (Stuhlman, 1979). Only certain strains of the Chinese species are affected, and the disease is probably transmitted by a recessive gene. The disease in the Chinese hamster has a rapid onset between 1 and 3 months of age. The indications of the disease are polyuria, polydipsia, glycosuria, and ketonuria. A normal Chinese hamster has glucose levels of 110 ± 6 mg/100 mL, whereas a diabetic hamster has levels of 200–800 mg/mL. The diabetes mellitus seen in the Chinese hamster is very similar to the disease state in humans. There is a variation in the syndromes (chronic, insulin dependent), the occurrence of secondary manifestations (cataracts), discrepancies in established parameters of native insulin values, and what role heredity plays in the disease.

In the South African hamster, which has a 22% incidence of diabetes mellitus, the disease is inherited as a non-sex-linked polygenic trait. With the South African hamster, the hyperglycemia varies in incidence, age of onset, degree of severity, and rate of progression, as in humans, but it is not influenced by age, sex, or state of obesity. Obesity is not a feature seen in the diabetic South African hamster, though it is seen in the Chinese hamster.

Either one of these models will provide insight into diabetes mellitus. These models can be used to study the disease pathogenesis, development of secondary complications, exact genetic mechanisms, and possible therapeutic regimens.

Leprosy

The hamster was first injected with leprosy bacilli in 1937. It was the first time a laboratory animal was found to be susceptible to the agent. In recent years, leprosy bacilli has been grown in cell culture systems and in the tail and foot pads of the mouse, so the role of the hamster in leprosy research has declined (Frenkel, 1987).

Muscular Dystrophy

The muscular dystrophy (MD) syndrome can be induced in hamsters by feeding them a diet deficient in vitamin E from weaning until death. The gross appearance of the disease state does not occur before the 11th or 12th month. Strains of cardiomyopathic Syrian hamsters (BIO 14.6 and BIO 53.38) do have genetic dispositions for this disease. The clinical signs appear 60–200 days after birth, and all skeletal muscles, including the heart, are affected. The lesions are pleomorphic, characterized by focal degeneration of myofibrils, coagulation necrosis, the formation of contraction clots, and alignment of muscle nuclei in chainlike rows within the fiber (Homburger and Bajusz, 1970). In the final stage of the syndrome, the myofibrils convert to granular mass in which the nuclei have disintegrated. Earliest changes occur at 33 days of age with death usually by 220 days due to cardiac failure. These hamsters have morphological manifestations of MD.

This model is excellent to study the mechanism of MD from an in vivo system to intact animals.

Osteoarthritis and Degenerative Joint Disease

Osteoarthritis and degenerative joint disease are diseases that a hamster may develop in old age. These diseases are rare in hamsters under 2 years of age. The diseases are characterized by separation of the zone of calcification of the cartilage with sclerosis and dislocation of the bone, fibrillation of ligaments, and fibrosis of the synovial membrane. Organisms such as *Mycoplasma*,

Streptobacillus moniliformis, *Corynebacterium kutscheri*, hormonal imbalances, and chemical and physical agents are associated with the disease. The disease in hamsters is similar to that seen in humans (Handler, 1965), which makes this an excellent model to study.

Pancreatic Cancer

Pancreatic cancer is a very difficult neoplasm to induce in laboratory animals. It can be induced in Syrian hamsters with nitrosamines. The neoplasms, histogenesis, and enzymatic patterns seen in human pathogenesis are similar to those in the hamster. Occasionally, hamsters develop diabetes during the pancreatic carcinogenesis, as is also seen in the pathogenesis in humans.

PATHOLOGY

Frederick G. Hess

Background: Milestones in the Hamster Life Cycle

A newborn Syrian hamster *Mesocricetus auratus* weighs approximately 2–3 g; an adult of 6 months weighs approximately 150 g. For males, spermatogenesis commences at approximately day 25 of age; sexual maturity is reached at day 40 for females, and earliest ovulation occurs from weeks 4 to 8. The average estrus cycle is 4 days, whereas gestation occurs in 16 days.

Litters vary in size from 4 to 12 pups. Females of some strains are able to produce six litters or more (Van Hoosier and Ladiges, 1984). Normal weaning time is 21–28 days. Breeding capability for males and females is approximately 12 months.

The average life span for these naturally nocturnal animals is approximately 2 years, with a 3-year maximum life expectancy (Van G Hoosier and Ladiges, 1984). However, specific closed colonies have had mean survival times of 63–79 weeks for males and 50–63 weeks for females (Pour et al., 1979; Redman et al., 1979; Slauson et al., 1978), whereas another colony had a mean survival time of 106 weeks for males and 97 weeks for females (Kamino et al., 2001a,b).

Special Anatomical Features

Anatomically, a unique morphological feature of the Syrian hamster is its well-developed cheek pouches, which are highly distensible evaginations of the lateral buccal walls. The hamster utilizes them to transport and store food. For experimental purposes, these readily accessible structures are eversible and may be utilized as sites for normal/abnormal tissue transplants (including tumor implantations). Immunological rejection does not occur owing to the absence of an intact lymphatic drainage pathway (Van Hoosier and Ladiges, 1984).

The upper gastrointestinal tract is unusual in that the esophagus enters between the forestomach and glandular stomach. Also at this junction, the limiting ridge of the hamster stomach represents a distinct constriction, forming the two separate compartments.

Nonneoplastic Lesions

The published incidence rates of the most frequent nonneoplastic lesions of untreated outbred Syrian hamsters are listed in Table 4.11. Since most of these lesions were tabulated using only one or two references and proper historical control data have not been published, the actual incidences are probably lower than those given in Table 4.11. Various factors, which are known to increase

Table 4.11   Incidence of Various Nonneoplastic Lesions in Untreated Outbred Syrian (Golden) Hamsters

Organ	Lesion	% Incidence in Males	% Incidence in Females	References
Adrenal glands	Cortical cyst	2	8	Pour et al. (1976c)
	Cortical hyperplasia	10–72a	5–61a	Pour et al. (1976c); Deamond et al. (1990)
				Kamino et al. (2001a)
Arteries	Vascular calcinosis	5–13	10	Pour et al. (1976a)
Colon/cecum/ileum	Enteritis (colitis/typhlitis/ proliferative ileitis)	10–73	20–73	Pour et al. (1976a,b) Frisk and Wagner (1977a,b)
Heart	Thrombosis	10–73	20–73	Pour et al. (1976a)
				McMartin (1977)
				McMartin and Dodds (1982); Deamond et al. (1990)
Kidneys	Arteriolar nephrosclerosis	72	80	Slauson et al. (1978)
	Nephrocalcinosis	15–31	16–20	Slauson et al. (1978) Deamond et al. (1990)
	Calcification	63a	47a	Kamino et al. (2001a)
	Infarct	42a	47a	Kamino et al. (2001a)
	Pyelonephritis, acute/chronic	6–13	8–13	Pour et al. (1976a)
Liver	Cholangiectasis/ cystic change	21–60a	12–70a	Pour et al. (1976b); Deamand et al. (1990); Kamino et al. (2001a)
	Cholangitis	30–49	29–31	Pour et al. (1976a,b)
	Clear cell foci fatty change	— 51a	35a 59a	Kamino et al. (2001a)
Pancreas	Islet cell hyperplasia	37–64	30–81	Pour et al. (1976c)
Parathyroid glands	Hyperplasia (principal cells)	83	22	Pour et al. (1976c)
Ovaries	Cyst	—	6	Pour et al. (1976c)
Sternum	Cartilage degeneration	97a	97a	Kamino et al. (2001a)
Stomach/(forestomach/ glandular-pylorus region)	Erosions/ulcerations	4–8	5–20	Pour et al. (1976a,b)
Testes	Tubular atrophy	40a	—	Kamino et al. (2001a)
Uterus	Granular cell foci	—	54a	Kamino et al. (2001a)
Vertebra	Chondrosis	30–83	10–81	Pour et al. (1976a)
Multiple organs	Amyloidosis	16–88	16–88	Gleiser et al. (1971); Pour et al. (1976a); Deamand et al. (1990); Kamino et al. (2001a)

Note: Maximal incidence rate includes animals surviving up to 33 weeks of age.

frequency rates, include age, sex, diet, breeding methods, genetic drift, hormonal imbalance, the presence of transmissible bacterial, viral, or parasitic agents, and the extent of gross necropsy and histopathological evaluations.

Amyloidosis

Systemic amyloidosis is a principal cause of death in aging hamsters (Van Hoosier and Ladiges, 1984). In one colony, a high frequency of 88% occurred in hamsters over 18 months of age and a

lower frequency 42% in hamsters 13–18 months of age (Gleiser et al., 1971). In another colony, systematic amyloidosis was noted as the cause of death of approximately 25% of males that were sacrificed at week 53 and 33% of females that were sacrificed at week 40 (Slauson et al., 1978).

By analysis of serum electrophoretic patterns, there was a decrease in albumin and a concomitant increase in total globulin, primarily a sharp transient rise in α2 serum globulin, as detected in 12-month-old hamsters (Van Koosier and Ladiges, 1984). By transmission electron microscopy, amyloid deposits were identified as finely granular amorphous material that was located in the mesangial matrix and between endothelial cells and the basement membranes of renal glomeruli.

By light microscopy (LM), the initial lesion appears to be located in the walls of glomerular capillaries, forming so-called wire-loop lesions. In addition, amyloid accumulation in epithelium/connective tissue has been seen primarily in hepatic periportal areas, adrenal cortex, pancreas, and adjacent to splenic lymphoid follicles. Less frequent sites are the lungs, ovaries, testes, and epididymides.

For positive diagnosis by LM, Congo red stain and the birefringence of these areas in polarized light are utilized (Gleiser et al., 1971). Biochemically, two proteins, namely, amyloid A (AA) (major component) and amyloid P (AP) (minor component), have been identified in isolated fibrils from aged hamsters (Brandwein et al., 1981; Coe and Ross, 1990). Only the AP component is under sex hormone control in the Syrian hamster, which distinctly predisposes females to acquire amyloidosis normally with aging. Testosterone inhibits the hepatic synthesis of the AP component homologue (called female protein), which is normally expressed from 100- to 200-fold times greater in the female Syrian Hamster, as compared to the male (Coe and Ross, 1990).

Arteriolar Nephrosclerosis

Arteriolar nephrosclerosis was observed at approximately 75% incidence of control animals (Slauson et al., 1978). Progressive renal arteriolar sclerosis with subsequent glomerulosclerosis (fibrinoid necrosis) and tubular degeneration/atrophy with fibrosis were described. Degenerative vascular changes (fibrinoid necrosis) were seen in the testes, ovaries, and uterus. Although limited serological analyses for anti-LCM antibody were negative, chronic viral infection was considered as the potential cause of the disease.

Atrial Thrombosis

Certain colonies of Syrian hamsters have been maintained and utilized as prospective animal models. For instances, a high incidence (73%) of aging male and female acromelanic Syrian hamsters had atrial thrombosis, accompanied by a consumption coagulopathy (McMartin, 1977; McMartin and Dodds, 1982).

The thrombi were located primarily in the left atrium, resulting from localized hemostasis secondary to cardiac failure. Atrioventricular valvular thickening and bilateral ventricular hypertrophy were often present. Pulmonary edema and pleural effusion were commonly seen at gross necropsy, which correlated clinically to hyperpnea, tachycardia, and cyanosis. Microscopically, myocardial degeneration/necrosis was associated with calcification and fibrosis. Similar cardiac lesions were noted at a frequency of 40% per sex in a closed colony of APA strain, random-bred hamsters (Doi et al., 1987). Glomerulonephrosis was also noted with no apparent correlation to the cardiac thrombosis as well as chronic renal disease characterized by tubular dilation/atrophy and proteinaceous casts.

A cardiomyopathic hamster model with progression to myocardial failure has been described (Bajusz et al., 1969; Gertz, 1973). Within 65 days of age, hamsters of both sexes of the inbred strain BIO 14.6 developed myocardial degeneration/necrosis with calcification as well as generalized myodystrophy. At 9–12 months of age, marked ventricular hypertrophy, thrombosis of the left atrium, and hepatic chronic passive congestion were seen, indicative of an animal model for congestive heart failure.

Generalized Vascular Calcinosis

Generalized vascular calcinosis was noted in the aorta and coronary and renal arteries (Pour et al., 1976a). Later, this abnormality was ameliorated by modifications in the diet (Birt and Pour, 1983; Pour and Birt, 1979).

Hemorrhagic Necrosis

In the late 1970s, spontaneous hemorrhagic necrosis (SHN) was described in late-term fetal hamsters at days 14–15 of gestation (Keeler and Young, 1978; Young and Keeler, 1978). It was characterized by multifocal lesions with coalescent zones of parenchymal hemorrhage, edema, and necrosis affecting the subependymal capillary vasculature of the forebrain, thalamus, medulla, and spinal cord. Litter viability was decreased.

Fetal brain development and differentiation were influenced by intrauterine environmental factors, especially diet deficient in vitamin E. Supplementation of diet with vitamin E throughout gestation and lactation completely prevented SHN and fully restored litter viability to normal levels (Keeler and Young, 1979). Crossbreeding of susceptible/non-susceptible strains eliminated the disease (Keeler and Young, 1978).

Bacterial Infections

Hamster Enteritis

Several important bacterial infectious agents have been known to affect the health status of the Syrian hamster as well as to produce complications in histopathological evaluations. Foremost, HE remains a disease entity of great concern. It is usually manifested early as an epizootic disease with a high mortality of approximately 90% of weanling hamsters from 3 to 8 weeks of age (Frisk and Wagner, 1977a).

In this chapter, hamster enteritis will be the term used to describe wet tail, or wet tail disease, which also has been referred to as proliferate ileitis, regional enteritis, terminal ileitis, atypical (transmissible) ileal hyperplasia, and enzootic intestinal adenocarcinoma (with localized invasion to adjacent muscularis). HE appears to include a spectrum of various stages of these lesions.

Clinical symptoms/signs include diarrhea, dehydration, lethargy, anorexia, and irritability. The disease progresses to staining of the perineum, tail, and ventral abdomen. Death occurs from 48 hours to 1 week after the onset of symptoms.

Gross necropsy lesions range from mild acute changes such as ileal hyperemia/hemorrhage to marked distension of segments of the ileum. Mucosal areas may become ulcerative and necrotic, eventually penetrating into the muscular layers, resulting in multiple abscesses of the subserosa. Enlargement of the mesenteric lymph nodes and peritonitis with adhesions also develop. Intussusceptions of the colon and prolepses of the rectum have been described for hamsters that recovered from HE (Jacoby et al., 1975).

Microscopic lesions range from mild acute enteritis with epithelial hyperplasia of the columnar cells to marked epithelial hyperplasia associated with chronic inflammation and fibrosis. Other regions of the mucosa have epithelial degeneration/necrosis and hemorrhages, ulcerations, and/or abscesses (Frisk and Wagner, 1977a). Associated lesions include lymphadenitis of the mesenteric lymph nodes (Amend et al., 1976).

Etiologically, intracytoplasmic bacteria have been demonstrated by transmission electron microscopy (Frisk et al., 1981; Frisk and Wagner, 1977a; Johnson and Jacoby, 1978; Wagner et al., 1973).

Intracytoplasmic particles resembling rod-shaped bacteria have been detected with indirect fluorescent-antibody technique using serum from hamsters with HE (Frisk et al., 1981; Jacoby, 1978; Jacoby et al., 1975).

Specifically, the bacterium *E. coli* has been isolated from the intestinal tract of hamsters with HE (Amend et al., 1976; Frisk et al., 1978, 1981; McNeil et al., 1986). In one facility, *E. coli* was isolated early in the disease process, whereas *Campylobacter* (Dillehay et al., 1994) was observed in later stages within hyperplastic epithelial cells (Frisk and Wagner, 1977a). In addition, Jacoby (1978) demonstrated by immunofluorescence an intracellular antigen morphologically compatible with gram-negative rods like *Campylobacter fetus*, whereas Dillehay et al. (1994) demonstrated campylobacter-like organisms by transmission electron microscopy (TEM). Thus, two or more organisms may act synergistically to produce the disease, since neither *E. coli* nor *C. fetus* cultured alone could reproduce the natural disease (Van Hoosier and Ladiges, 1984).

Similar gross and microscopic changes as described earlier for HE were noted predominately in the ceca of Syrian hamsters (Barthold and Smith, 1984; Rehg and Lu, 1982).

The most effective treatment for HE has been oxytetracycline in the drinking water, as compared to neomycin, dimetrdazole, and tetracycline hydrochloride (McNeil et al., 1986). Other antimicrobials, i.e., clindamycin, lincomycin, ampicillin, vancomycin, erythromycin, cephalosporins, and gentamicin, caused fatal gastrointestinal toxicity to hamsters with clinical symptoms, including diarrhea, dehydration, anorexia, and hypothermia (Bartlett et al., 1978; Hawkins et al., 1984; Lusk et al., 1978). In another report, effective treatment was demonstrated by vancomycin hydrochloride, which did not cause major adverse effects (Boss et al., 1994).

The anaerobe *Clostridium difficile* was found to be the direct cause of the enterocolitis producing overgrowth of the ileal/colonic/cecal flora. Gross pathology consisted of distension, hyperemia, and hemorrhage of the ileum, cecum, and ascending colon. Histological sections revealed mucosal acute and chronic inflammation and congestion/hemorrhages as well as luminal collections of inflammatory cells, mucin, and sloughed epithelial cells.

Tyzzer's Disease

As compared to HE, Tyzzer's disease has been noted much less frequently. The only outbreak of Tyzzer's disease in a colony bred in the United States was reported in 1977 (Zook et al., 1977). Two other epizootics were reported in Japan (Nakayama et al., 1975; Takasaki et al., 1974).

Clinical symptoms include the sudden onset of diarrhea, dehydration, and lethargy; deaths occur within 48 hours. At gross necropsy, the most common lesion is yellow-white foci (1–2 mm diameter) in the liver, corresponding microscopically to areas of nectosis with inflammatory infiltrates. Secondary changes are thickened and discolored portions of the terminal ileum, cecum, and colon. These regions correspond microscopically to coagulative necrosis associated with epithelial cell sloughing, submucosal edema, polymorphonuclear and mononuclear cellular infiltrates, multinucleated giant cells, and fibrosis.

The causative agent is a gram-negative pleomorphic bacterium, *Bacillus piliformis*. Positive diagnosis has been demonstrated with PAS and Giemsa stain within the cytoplasm of intestinal epithelial cells as well as hepatocytes and myocardial fibers.

Other Bacterial Infections

The possible roles of bacteria (e.g., *Pasteurella pneumotropica* and *Streptococcus pneumoniae*) as well as *Mycoplasma pulmonis* in the etiology of pneumonia in Syrian hamsters have not been clearly defined (Van Hoosier and Ladiges, 1984).

Viral Infections

Lymphocytic Choriomeningitis

Syrian hamsters and contaminated tumor cell lines were likely sources of the infectious RNA LCM virus, which infected various laboratory personnel between 1965 and 1974, as well as other naive hamsters (Biggar et al., 1977; Skinner and Knight, 1979). Commercial distribution of the cell lines ceased in 1974.

Clinical signs/symptoms of LCM occur rarely in hamsters, even in those with chronic infections for months. However, ataxia, conjunctivitis, dehydration, and tremors have been noted in a few hamsters. Death occurs following chronic progressive infection characterized by viremia, viruria, and high titers of virus in tissues (Parker et al., 1976). Experimentally, complement-fixing antibodies have been detected in young adult hamsters by 10 days after inoculation.

Microscopic lesions include conjunctivitis, chronic glomerulonephritis, generalized vasculitis, and lymphocytic cellular infiltrates in the liver and kidneys.

Sendai Virus

Parainfluenza 1 (Sendai) is an RNA agent of the paramyxovirus group that has been implicated as a cause of hamster pneumonia. An enzootic form of the disease was reported at a research facility (Profeta et al., 1969). At gross necropsy, complete consolidation of the lungs has been observed. Occasional deaths have been reported in suckling hamsters (Van Hoosier and Ladiges, 1984).

Adenovirus

Syrian hamsters from 10 production colonies, including major commercial suppliers in the United States, had subclinical adenoviral infections (Gibson et al., 1990). Serum antibodies reacted with mouse adenovirus strains K87 and FL by indirect fluorescence, which correlated to the presence of intranuclear inclusion bodies in ileal enterocytes by LM and TEM.

Fungal Infections

Hamsters are susceptible to various fungal infections. Histoplasmosis, for example, has been studied extensively; hamsters are highly sensitive and, therefore, useful for diagnostic purposes. Most fungi grow in the spleen, lymph nodes, and liver (Van Hoosier and Ladiges, 1984).

Parasitic Infections

Cestodes

Hymenolepis nana, the dwarf tapeworm, is the most prevalent internal parasite found in hamsters (Van Hoosier and Ladiges, 1984). Their size varies from 25 to 40 mm in length, and they are usually found in the small intestine.

Although the consequences of infection are usually benign, impactions followed by deaths have been observed, dependent on the number of parasites and degree of intestinal occlusion.

Preventive measures rely on routine sanitation of cages and effective insect and vermin control. Yomesan (niclosamide) has been reported as effective and safe for the treatment of cestode infections in hamsters (Ronald and Wagner, 1975). Laboratory personnel should be aware of the possible transmissible potential and receive proper hygienic procedures.

Mites

Two species of mites (*Demodex aurati* and *D. criceti)* have been identified in hamster epidermis (Flatt and Kerber, 1968; Owen and Young, 1973). *D. aurati* resides in hair follicles, whereas *D. criceti* inhabits folds in the epidermis.

Both species appear to be asymptomatic, low-grade pathogens. However, clinical skin disease in which *D. aurati* was identified resulted in demodectic mange consisting of marked alopecia and scab formation in 50% incidence of male hamsters (Estes et al., 1971). Predisposing factors included sex, age, and stress.

Neoplastic Lesions

The incidence rates of naturally occurring tumors in untreated outbred Syrian hamsters are generally low. The most frequent tumor types are listed in Table 4.12, as reported in references from 1970 to 2001. Significant low spontaneous tumor rates (<2%) are noted for the respiratory tract, pituitary gland, and mammary gland of both sexes and the genital tract of male hamsters. Such low incidences establish the Syrian hamster as a good candidate for chemical carcinogenicity studies, including morphological studies utilizing electron microscopy, cytology, and histochemistry (Becci et al., 1978; Hess et al., 1981a,b; Schreiber et al., 1974).

Table 4.12   Incidence of Various Neoplasms in Untreated Outbred Syrian (Golden) Hamsters

Organ	Tumor Type	% Incidence in Males	% Incidence in Females	References
Adrenal glands	Cortical adenoma	10–49a	2–23b	Pour et al. (1976c)
	Cortical carcinoma, Pheochromocytoma	3	2	Pour et al. (1976c); Kamino et al. (2001b)
Colon/cecum	Adenocarcinoma (a)	7	20	Fabry (1985)
Harderian gland	Adenoma	3	—	Pour et al. (1976b)
Liver	Cholangioma	4	7	Pour et al. (1976b)
	Hepatocellular adenoma	4	0	Pour et al. (1976b)
Lymphoreticular system	Malignant lymphoma (lymph sarcoma)	2–53	2–53	Ambrose and Coggin (1975); Pour et al. (1976c)
				Coggin et al. (1983); Kamino et al. (2001b)
Ovaries	Granular cell tumor/ thecoma	—	3–4	Kamino et al. (2001b); Pour et al. (1976c)
Pancreas	Islet cell adenoma	4–14	2–3	Kamino et al. (2001b); Pour et al. (1976c)
Parathyroid glands	Adenoma	2–5	4–7	Kamino et al. (2001b); Pour et al. (1976c)
Pituitary gland	Adenoma	—	6	Kamino et al. (2001b)
	Adenocarcinoma	—	3	Kamino et al. (2001b)
Skin	Melanoma	2	2	Turusov (1982)
Spleen	Hemangioendothelioma	1–3	—	Pour et al. (1976d)
Stomach (forestomach)	Papilloma	5–8	3–5	Pour et al. (1976d); Kamino et al. (2001b)
Uterus	Endometrial carcinoma	—	5–7	Kamino et al. (2001b); Pour et al. (1976c)
	Endometrial polyp	—	12	Pour et al. (1976c)
	Leiomyoma	—	3–10	Kamino et al. (2001b); Pour et al. (1976c)
Vagina	Papilloma	—	4	Pour et al. (1976c)

Since most of the tumor types in Table 4.12 are listed with only one or two references and factual historical data have not been published, the actual incidence rates are probably lower than those given. Various factors that cause these differences include diet, water quality, survival age, sex, breeding methods, genetic drift, hormonal imbalances, extent of gross necropsy and microscopic examinations, and the presence of transmissible agents.

One or more of these predisposing factors may become relatively predominant and complicate the frequency of historical tumor data. For example, in one report, the majority (75%) of lymph sarcomas was horizontally transmitted, suggesting that infectious agent (s) were involved (Van Hoodier and Trentin, 1979). Similarly, five epizootics of poorly differentiated lymphocytic lymphomas (53% incidence) were horizontally transmitted in two facilities by a vitriolic agent (Coggin et al., 1983). The predominant sites were the large and small intestines with involvement of mesenteric and cervical lymph nodes.

METABOLISM

While comparative studies of hamster and human metabolism are not extensive, some such studies exist (such as Young et al., 2001, for methyl mercury metabolism and deposition, Rockwood et al., 2003 for methemoglobin reductase, and Cheema and Cornish, 2007, for lipid metabolism), and there are, as we shall see, sufficient data to evaluate comparative metabolism.

Hepatic Microsomal Mixed-Function Oxidase

Most of the work pertaining to xenobiotic metabolism has concentrated on the Syrian or Syrian golden strain, with some on the Chinese. Therefore, most of the following discussion is based on this particular strain. As in all other species studied, the liver is the major site, and the microsomal mixed-function oxidase (MMFO) system is the major system involved in xenobiotic metabolism in the hamster. Identified P450 isoform activities are listed in Table 4.13, while some of the salient enzyme activities are summarized in Table 4.14. The liver-to-body-weight ratio in the nonfasted hamster is slightly higher than that of the rat and may range from 3.5% to 5.4% depending on sex, age, and protein content of the diet (Birt et al., 1983). CYP-450 concentrations are higher than those of rats, ranging between 0.95 and 1.35 nmol/mg microsomal protein in

Table 4.13 Specific CYP Activities Identified in the Hamster Isoform

CYP	1A1
CYP	1A2
CYP	2A8
CYP	2A9
CYP	2C25
CYP	2C26
CYP	2D20
CYP	2D27
CYP	2E1
CYP	3A10
CYP	7A1
CYP	11A1 (11B-hydroxylase and 19-hydroxylase but not alelosterone synthane-Veronneau et al., 1996)
CYP	17A1

Sources: Nelson, D. R. et al., *Pharmacogenetics*, 6, 1, 1996; Sagami, I. et al., *J. Biochem.*, 110, 641, 1991.

Table 4.14   Summary of Hepatic Xenobiotic Metabolizing Enzymes in Hamsters

Enzyme	Concentration or Activity	Comments and References
Cytochrome P-450	0.95–1.35 nmol/mg	Burke and Prough (1976), Smith et al. (1986); Ardies et al. (1987), Mc
		McCoy and Koop (1988); Blaich et al. (1988)
Cytochrome b_5	0.43–0.58 nmol/mg	Blaich et al. (1988); DeMarco and McCoy (1985); Smith et al. (1986)
NADPH: cytochrome P-450 reductase	200–320 nmol/min/mg	Blaich et al. (1988); Burke and Prough (1976); Smith et al. (1986)
MMFO activities		
Aniline	0.50–1.2 nmol/min/mg	Burke and Prough (1976); Fuji et al. (1985);
Benzphetamine	6.0–12.5 nmol/min/mg	Smith et al. (1986); Ardies et al. (1987);
7-Ethoxycoumarin	5.0–14 nmol/min/mg	McCoy and Koop (1988); Blaich et al. (1988)
Benzo(a)pyrene	0.3–2.5 nmol/min/mg	
Epoxide hydrolase (with styrene oxide) microsomal	12.6–25.9 nmol/min/mg	Pacifici et al. (1981); Oesch and Woolf (1989)
UDP-Glucuronosyl transferase (with 4-nitrophenol)	40–50 nmol/min/mg	Hietanen and Vainio (1976) (untreated microsomes; no trypsin or detergents)
Glutathione S-transferase (with chlorodinitrobenzene)		Igarashi et al. (1986), Lam (1988)
Cytosolic	7–8 µmol/min/mg	Morgenstern et al. (1984)
Microsomal	0.9–1.0 µmol/min/mg	
Protein estimates		Birt et al. (1983)
Microsomal	25–35 mg/g liver	Lechner and Gomes (1975)

Note: mg, mg protein.

most published reports. A concentration of 1.85 nmol/mg (for naive hamsters) has been reported (Chaing and Steggles, 1983). As in all species, cytochrome P-450 in hamsters exists as a family of isozymes, but the number of different isozymes has not been as well characterized in the hamster as in other rodent species. Nelson et al. (1996) and Sagami et al. (1991) have identified 13 different cytochrome P-450 isozymes. Concentrations of cytochrome P-450 also tend to be somewhat higher than those of the rat, ranging from 0.43 to 0.58 nmol/mg. The activity of NADPH: cytochrome C reductase is in the same range as that of the rat, i.e., 200–320 nmol/min/mg. It has not been established if this enzyme in the hamster plays the rate-limiting role in MMFO activity that it does in the rat.

Metabolism of the cytochrome P-450-dependent monooxygenase, when evaluated in hamster liver microsomes by measuring aniline hydroxylation, benzylphelamine demethylation, and benzo (a) pyrene hydroxylation, defluaranation of enflurane was evaluated by measuring free fluoride metabolites. All were inducible with in vivo treatment of animals suggesting that pure in vitro effects are of limited value in predicting in vivo metabolism (Chen et al., 1995). Furthermore, many of the inform activities are not limited to the liver but are also present in the lungs and other tissues (Sagami et al., 1991). Ohhira et al. (1999) have demonstrated phenobarbital induction of CYP isoenzyme metabolism of triphenyltin.

The saturating activities of the hamster MMFO with various common model substrates are also summarized in Table 4.14. In general, activity tends to be higher than that of the rat. Few differences in qualitative species MMFO substrate selectivity have been identified between the rat and the hamster. There are interesting and important quantitative differences. For example, the fact that carbon tetrachloride has been reported to be a hepatocarcinogen in hamsters but not in rats is apparently due to differences in the rates of metabolism. Castro et al. (1989) have demonstrated that hamsters have a much higher rate (both in vivo and in vitro) of production of reactive metabolites

that bind to nuclear protein than rats. Weyand and Bevan (1987) examined the in vivo disposition of benzo(a)pyrene in different rodent species. The major difference between the hamster and the other species was that increased amounts of radioactivity were retained in the lungs of hamsters at lower doses with a proportional decrease in the amount of radioactivity excreted into the bile. Bergman et al. (1984) studied species differences in a-naphthoflavone (ANF) microsomal metabolism. Total metabolism was essentially the same in rats and hamsters, and the same three chemicals made up the majority of the metabolites in both species (ANF-5,6-oxide, ANF-6-phenol, and ANF-7,8-dihydrodiol). The oxide, however, was the major metabolite in rats, and the dihydrodiol was the major metabolite in hamsters, indicating a site-selective species difference.

Birt et al. (1983) studied the effects of age, gender, and dietary protein on the hepatic MMFO in hamsters. In general, a high-protein diet (10% vs 40% lactalbumin) tended to cause slight increases in the levels of microsomal protein and tended to cause the most pronounced sex- and age-related differences. When the results of only the low-protein diet (which would be most representative of standard hamster chow) were evaluated, some interesting observations become apparent. In general, microsomal protein tended to increase as a function of age, while MMFO activity either increased or remained about the same in comparing activity in young versus old hamsters. While a few isolated differences were identified, consistent and convincing differences between sexes were not demonstrated. Cytochrome P-450 also tended to increase as a function of both dietary protein content and age. Changes in cytochrome P-450, however, were not reflected in equivalent increases in MMFO activity. For example, in 18-week-old female rats (10% lactoglobulin), the cytochrome P-450 concentration was 0.58 nmol/mg protein and aromatic hydrocarbon hydroxylase (AHH) activity was 132 ng/min/mg, whereas in 60-week-old rats (40% lactoglobulin), cytochrome P-450 content was 1.62 nmol/mg and AHH activity was 126 ng/mg/min. This suggests the possibility that there are age- and/or dietary-related variations in the isozymic character of cytochrome P-450 in hamsters.

Microsomal Induction

As amply reviewed elsewhere, in vivo treatment of mammals with a wide variety of organic chemicals can result in increases in MMFO activity by a process called enzyme induction. The prototypical inducing agent is phenobarbital, and its effects in hamsters have been well documented. Lechner and Gomes (1975) reported that the treatment of hamsters with phenobarbital (80 mg/kg/day for 2 days, po) induced about a 20% increase in microsomal protein, a 75% increase in cytochrome P-450, and significant increase in MMFO activity with both aminopyrene and aniline. McCoy et al. (1981) compared the effect of both phenobarbital and 3-methylcholanthrene (3-MC) in both rats and hamsters. Both treatments caused increases in cytochrome P-450 in both species, although there were slight quantitative differences in the amount induced. When benzo(a)pyrene metabolism was examined, however, there was a marked difference in species response: phenobarbital increased metabolism in hamsters, but 3-MC did not, whereas phenobarbital did not increase this activity in rats, but 3-MC caused a two orders of magnitude increase. Chiang and Steggles (1983) used an inducing regimen of a single ip dose of phenobarbital (50 mg/kg) followed by 4 days of treatment with 0.1% phenobarbital in the drinking water. This resulted in a 40% increase in cytochrome P-450. The MMFO activity on a milligram protein basis was significantly increased with all substrates, with benzphetamine and 7-ethoxycoumarin activity on a nanomole P-450 basis was significantly increased as well. Smith et al. (1986) treated hamsters with phenobarbital (70 mg/kg ip for 4 days) and observed a 65% increase in cytochrome P-450 as well as a significant increase in cytochrome b5 that were accompanied by increases in MMFO activity toward both benzphetamine and ethoxycoumarin. This increase in cytochrome b5, however, has not been confirmed by other investigators (Blaich et al., 1988). Blaich et al. (1988) directly compared the inducing effects of phenobarbital on MMFO activity of both rats and hamsters. As expected, significant induction of activity toward ethoxycoumarin (the prototypical substrate for the isozyme of cytochrome

P-450x induced in the rat by phenobarbital) was obtained in both species. In contrast, phenobarbital induced slight increases in rats toward both benzo(a)pyrene and ethoxyresorufin (substrates of P-448 induced by 3-MC in rats), whereas in hamsters, phenobarbital increased activity only toward benzo(a)pyrene. Hence, phenobarbital is an effective inducing agent in hamsters, but the response of hamsters is somewhat different from that in rats. In general, phenobarbital effectively induces the activity in both species toward substrates, such as benzphetamine and ethoxycoumarin, of rat cytochrome isozyme P-450e. In contrast, phenobarbital and 3-MC are equally efficacious in hamsters in inducing increases in the metabolism of substrates, such as benzo(a)pyrene, of rat cytochrome isozyme P-450d (P-448). As will be detailed further, hamsters and rats differ considerably in their induction with 3-MC-type agents.

The effect of traditional cytochrome P-450- or P-448-inducing agents has been studied in the hamster, and the response of the hamster to some of these agents has been shown to be different from that of the rat. Burke and Prough (1976) noted that treatment of hamsters with 20 mg/kg ip once daily for 3 days of 3-methylcholanthrene (a classic or prototype-inducing agent of cytochrome PI-450 in rats) did not increase MMFO activity toward benzo(a)pyrene, ethylmorphine, or benzphetamine, but did increase activity toward biphenyl 2.4- to 3.4-fold. The activity of NADPH: cytochrome P-450 reductase was not induced, and this is an observation that has been generally confirmed by most subsequent investigations on MMFO induction in the hamster.

Hietanen and Vainio (1976) examined the inducing effect of DDT (1,1,1-trichloro-2,2-bis([p-chlorophenyl)ethane) on in vitro benzo(a)pyrene metabolism in four different species, including the hamster. In terms of baseline activity, the animals had the following rank: guinea pig>hamster>mouse>rat. Treatment with DDT (single dose, 160 mg/kg po), however, increased the activity twofold in hamsters, whereas actually causing slight decreases in the other species. These early papers suggest that hamsters are relatively resistant to 3-MC induction, while still responding to halogenated aromatics. This was confirmed by Chiang and Steggles (1983), who noted that 3-MC induced increases in microsomal cytochrome P-450 (and thereby did induce some increases in MMFO activity on a milligram protein basis), but did not increase MMFO activity per nanomole of cytochrome P-450 toward benzphetamine, 7-ethoxycoumarin, benzo(a)pyrene, or p-nitrophenetole.

Blaich et al. (1988) also reported data that suggest that while 3-MC induces increases in microsomal benzo(a)pyrene metabolism, these increases seem to be due mostly to increases in total microsomal protein rather than a specific isozyme. Comparable treatment of rats with 3-MC induces much larger increases in microsomal benz(a)pyrene metabolism.

On the other hand, Chiang and Steggles (1983) reported that polychlorinated biphenyls (single dose, 300 mg/kg ip) induced large increases in cytochrome P-450 and had about the same effect on benzo(a)pyrene metabolism as 3-MC, but (unlike 3-MC) increased MMFO activity per nanomole cytochrome P-450 toward benzphetamine and p-nitrophenetole. Interestingly, 6-naphthalflavone (BNF), which in the rat is considered to be a 3-MC-type inducer, induced a different pattern in hamsters than 3-MC did in that it induced increases in microsomal 7-ethoxycoumarin metabolism, both on a milligram microsomal protein and on a nanomole cytochrome P-450 basis, but actually decreases benzphetamine metabolism (Ardies et al., 1987; Chiang and Steggles, 1983; Smith et al., 1986). Across the board, 7-ethoxyresorufin deethylation is induced by 3-MC, BNF, and polyhalogenated hydrocarbons in hamsters as it is in rats (Blaich et al., 1988; Chiang and Steggles, 1983; Smith et al., 1986). The response is much greater in rats; 3-MC induces a 30-fold increase in rats as opposed to a 3-fold increase in hamsters (Iwasaki et al., 1986). Thus, with regard to cytochrome PI-450 or P-448 induction in the hamster, three points should be kept in mind. First, 3-MC-type agents are less effective in inducing AHH-type activity in hamsters than in rats. Second, different agents of this class have more selective effects on MMFO activity than in rats (e.g., BNF induces activity different from 3-MC). Third, halogenated hydrocarbons are the most effective members of this class in inducing generalized increases in MMFO activities, and hamsters may, in fact, be the most sensitive rodent to these agents (e.g., DDT).

The prototypical type 3, or steroidal inducing agent, pregnenolone-16(a)-carbonitrile has no effect on MMFO activity in hamsters (Chiang and Steggles, 1983).

Sex-Related Differences

Unlike the situation in the rat, there are no consistently reported marked sex-related differences in cytochrome P-450 concentrations or in MMFO activity. There are some suggestive hints in the literature, however. Blaich et al. (1988) examined MMFO induction in male and female hamsters, and found that 3-MC increased the cytochrome P-450 content in both sexes: 1.34–2.27 nmol/mg for males and 1.24–2.58 nmol/mg for females. Cytochrome b5, in contrast, was increased only in females: from 0.43 to 0.48 in nmol/mg males and from 0.53 to 0.71 nmol/mg in females. As in the rat, MMFO induction was accompanied by large increases in ethoxyresorufin deethylation: from 0.14 to 1.16 nmol/min/mg for males and from 0.08 to 1.39 nmol/min/mg for females. With regard to ethoxycoumarin deethylation, however, activity was significantly increased in males (5.37–7.47 nmol/min/mg), but decreased in females (5.27–3.23 nmoVmin/mg). Phenobarbital had the same inducing effect regardless of the sex in hamsters.

What are the toxicological consequences of MMFO induction in hamsters? There are a few examples in the literature that can be discussed here. Blaich and Metzler (1988) studied the effects of microsomal enzyme induction on DES metabolism and toxicity in hamsters. They observed that pretreatment of male hamsters with 7,8-benzoflavone decreases the incidence of kidney tumors but increases the incidence of liver tumors.

Ioannides et al. (1981) compared baseline and induced (with phenobarbital and 3-MC) mutagen (benzo(a)pyrene and 2-acetylaminofluorene) activation of the hamster, guinea pig, mouse, and rat in the Ames assay, which uses the S9 liver fraction (a combination of cytosol and microsomes). In all three species, 3-MC induction increased the mutagenicity of benzo(a)pyrene, whereas phenobarbital decreases it. Given the previous discussion that phenobarbital and 3-MC have approximately the same effect on microsomal benzo(a)pyrene metabolism (Chiang and Steggles, 1983), these results would suggest that there are other factors involved in mutagenic microsomal metabolism.

Santhanam and Lotlikar (1989) reported that pretreatment of hamsters with BNF increased the extent of aflatoxin B, binding to hepatic deoxyribonucleic acid in vivo. They further reported that this response differentiates the hamster from the rat (in which BNF decreases aflatoxin "activation") and may make the hamster an attractive model to study aflatoxin-induced carcinogenesis.

Ethanol Induction and Metabolism

The role of the MMFO in ethanol metabolism is the subject of some debate. There is ample evidence to demonstrate, however, that ethanol induces a specific isozyme of cytochrome P-450. As it has been reported that the hamster more avidly drinks ethanol than the rat (Ardies et al., 1987), the hamster may be a more attractive model for the study of ethanol metabolism and toxicity. Thus, the inducing effect of ethanol in the hamster has been studied by several investigators.

DeMarco and McCoy (1985) compared the effect of two different 28-day ethanol treatment regimens (drinking water vs liquid diet) in hamsters. They observed that only the liquid diet regimen was effective in increasing cytochrome P-450 content, but this increase was accompanied by a decrease in cytochrome b5 content and benzo(a)pyrene hydroxylase activity.

Fuji et al. (1985) used the drinking water regimen (10% ethanol v/v for 3 weeks) and also noted that ethanol causes modest increases in cytochrome P-450 in hamsters, which was accompanied by decreases in MMFO activity (on a nanomole cytochrome P-450 basis) with benzphetamine, 7-ethoxycoumarin, and benzo(a)pyrene. In contrast, there were increases in aniline hydroxylation, DMN demethylation, and ethanol oxidation activity. As discussed by Fuji et al. (1985), these data are sufficient to conclude that ethanol induction in the hamster is different from that observed in the

rat, in which a large specific increase in 7-ethoxycoumarin deethylation occurs. These data also suggest that ethanol induces a specific isozyme of cytochrome P-450 at the expense of other isozymes in the hamster.

Ardies et al. (1987) compared the effect of ethanol with other inducing agents (phenobarbital, BNF, and isoniazid) in hamsters. The resultant MMFO of each inducing agent was distinctive with regard to the composite picture produced by the ethyl isocyanide–binding spectrum, induction of cytochrome b5 effects on activity with specific substrates, and pattern produced by SDS-PAGE on microsomal protein. Consistent with previous publications, the MMFO of ethanol-treated (10% v/v ethanol in drinking water) hamsters had increased activity with ethanol and aniline but not with benzphetamine or 7-ethoxycoumarin (Ardies et al., 1987).

Using both SDS-PAGE and immunoprotein methods, McCoy and Koop (1988) further confirmed the existence of a distinctive cytochrome P-450 induced by ethanol in hamsters. Hence, ethanol does induce a specific cytochrome P-450 in hamsters, which, depending on treatment regimen, may or may not be reflected by an increased total cytochrome P-450 that has a rather narrow substrate specificity.

TCDD Metabolism and Induction

Rat and hamsters display a 100-fold difference in sensitivity to 2,3,7,8-tetrachlorodibenzodioxin (TCDD). Hamsters are, in fact, among the least sensitive species studied, having an LD_{50} between 1 and 5 mg/kg to this chemical. This observation has stimulated work comparing and contrasting the metabolism and the inducing effect of TCDD in hamsters and rats. Wroblewski and Olson (1988) studied the metabolism and inducing effects of TCDD in isolated rat and hamster hepatocytes. Interestingly, hepatocytes isolated from uninduced animals of both species had approximately the same rates of TCDD metabolism, and pretreatment with both 3-MC (50 mg/kg ip for 3 days) and TDCC (single doses; 5 mg/kg in rats and 500 mg/kg in hamsters) caused approximately the same five- to sixfold increase in TCDD metabolism in both species. TCDD pretreatment, however, induced increases in benzo(a)pyrene metabolism in rats and not hamsters. The cytochrome P-450-specific inhibitors metyrapone and ANF both had inhibitory effects on TCDD metabolism in rats, with ANF being far more effective. In hamsters, metyrapone had no effect, and ANF had only a slight and transitory effect on TCDD metabolism. In an earlier work, Wroblewski et al. (1988) noted that neither 3-MC nor TCDD induced increases in benzo(a)pyrene metabolism (when expressed on nanomole cytochrome P-450 basis), but both cause large increases in ethoxyresorufin metabolism in the hamster. These data suggest that TCDD has different inductive effect in rats and hamsters, but do not readily support the hypothesis that the species differences in TCDD toxicity are due to differences in metabolism.

Peroxisomal Proliferation

Hepatomegaly is a commonly used indicator of enzyme induction. However, hepatomegaly can also accompany induced increases in hepatic peroxisomes. The basic biology of these agents has been reviewed elsewhere. While rats and mice are very sensitive to these agents, Lake et al. (1984) reported that hamsters were far less sensitive to such agents. For example, equivalent doses of clofibrate (500 mg/kg) induced an 18-fold increase in carnitine acetyltransferase activity (a standard peroxisomal marker) in rats, but only a 2.5-fold increase in hamsters. Other data presented in this paper were consistent with this observation. This group (Lake et al., 1989a,b) expanded upon these observations with studies on nafenopin. They observed, for example, that when rats, hamsters, and guinea pigs were treated under the same dosage regimen (50 mg/kg/day by gavage for 3 weeks), the resulting increases in palmitoyl-CoA transferase were 10-fold in rats, 1.5 in hamsters, and none in the guinea pig. Watanabe et al. (1989) obtained similar results in their studies on the species

differences in peroxisomal proliferation associated with bezafibrate. Thus, the hamster is far less sensitive to peroxisomal proliferating agents than the rat and, therefore, may be a more appropriate model than the rat in assessing the toxicity of peroxisomal proliferating agents.

Epoxide Hydrolase

Aromatic groups frequently are oxidized to arene oxides or epoxides by the MMFO, which are in turn inactivated by hydrolysis to dihydrodiols by epoxide hydrolase. This is an important enzyme that has been intensely studied in a wide variety of species, except for hamsters. Initial work by Pacifici et al. (1981) suggests that the hamster may have relatively high epoxide hydrolase activity (12.6 nmol/min/mg microsomal protein) in comparison to other rodent species (hamster = guinea pig > rat > mouse). In comparison to nonrodent species, the rankings were baboon > hamster = human > dog. Interestingly, the hamster had the highest activities of renal and pulmonary epoxide hydrolase. Oesch and Wolf (1989), in their studies on hexachloro-1, 3-butadiene metabolism, confirmed that hamsters had higher epoxide hydrolase activity than other rodent species but obtained a higher saturating activity than Pacifici et al. (1981), i.e., 25.9 nmol/min/mg. Rigorous exploration of the substrate specificities, inhibitors, and other enzymological aspects of this enzyme in hamsters remains to be done.

Aromatic Amine Oxidation

Hamsters have proven to be convenient species for studying the oxidation of aromatic (primary or secondary) amines and amides. Lotlikar et al. (1967) noted that of the five most studied rodents (rat, hamster, mouse, rabbit, and guinea pig), hamsters had the highest baseline activity in the in vitro N-hydroxylation of 2-acetylaminofluorene and that such activity was highly inducible by pretreatment with 3-MC. Razzouk and Roberfroid (1982) confirmed that the hamster had higher activity than the rat with 2-AAF (and 2-aminofluorene), but observed that the mouse had higher activity than the hamster. The difference between the two papers may be due to the difference in mouse strains examined. The Razzouk paper did not specifically examine the relative contributions of the MMFO versus the flavine mixed-function oxidase (FMFO) system N-hydroxylation in the hamster, but did present data from experiments using inhibitors that the FMFO was at least partially involved.

Beckett and Gibson (1975) examined the species-related differences in N-hydroxylation of dibenzylamine and noted (consistent with Lotlikar et al., 1967 and Razzouk and Roberfroid, 1982) that the hamster had higher activity than either the rat or the mouse (but not as high as the rabbit or the guinea pig).

McMahon et al. (1980) examined N-hydroxylation of 4-aminobiphenyl in various species. N-Hydroxybiphenyl is the major metabolite of this chemical in the rat, guinea pig, C3H mouse, and hamster. The highest initial rates (in vitro) were seen in the hamster, whereas the lowest were seen in the rat. In contrast to the previous discussion on induction, these authors also reported that PCB treatment induced large increases in N-hydroxylation in the rat but had no effect in the hamster.

Gorrod and coworkers (1983, 1987) studied the metabolism of N-benzyl-4-substituted anilines and observed that hamsters had the highest N-hydroxylation activity, although it should be mentioned that the hamster also had the highest N-debenzylation activity. As discussed by Gemborys and Mudge (1981), the hamster has a high rate of N-hydroxylation with acetaminophen, which contributes to the sensitivity of this species to acetaminophen hepatotoxicity. Little work has been reported on the FMFO in hamsters, but the data reported by Gorrod and Patterson are consistent with the hypothesis that the FMFO is largely responsible for N-hydroxylation in the hamster.

Ioannides et al. (1981), in their studies on the effects of different inducing agents on the activation of 2-acetylaminofluorene, concluded that a system other than the MMFO is involved in this

process in hamsters as well as in other species. McCoy et al. (1986) studied the microsomal metabolism of nicotine in hamsters. Nicotine N′-oxide was one of the major metabolites in their studies, and using a combination of inhibitor and protease treatments, they were able to conclude that this reaction was mediated via the FMFO. In contrast, Nwosu and Crooks (1988) were unable to detect any nicotine N′-oxide as a urinary metabolite when nicotine was administered in vivo to hamsters. The reasons for the differences between these two papers are not evident, but the N-oxide could either be further metabolized or simply not be excreted via the urine. In general, when the combined literature is considered, the hamster has a relatively high capacity, especially compared to the rat, for the oxidation of aromatic amines and amides, and this activity is mediated, at least in part, by the FMFO.

N-Acetylation

The acetylation of aromatic amines to aromatic amides is generally considered to be the first step in the activation of aromatic amines to hepatocarcinogens (e.g., the conversion of 2-aminofluorene to 2-acetylaminofluorene). Lower and Bryan (1973) reported that the hamster has higher in vitro rates of aromatic amine acetylation than the guinea pig, mouse, and rat. For example, with 4-aminobiphenyl, the activity in hamster cytosolic preparations was 2.4-fold higher than that of the rat. Ioannides et al. (1981) compared the metabolic activation of 2-acetylaminofluorene to mutagens by the microsomes from different species. Hamster microsomes were extremely active in this regard, whereas rat microsomes displayed very weak activity. Given that the hamster has greater activity in the acetylation of aromatic amines and the subsequent oxidative activation of these to mutagenic intermediates, the hamster would probably be a better model than the rat in the study of the metabolism and toxicity of aromatic amines.

Interestingly, homozygous "rapid" and "slow" acetylator inbred strains of hamster have been identified and characterized (Hien et al., 1986; Trinidad et al., 1989). The slow acetylator strain has only 3% of the N-acetyl transferase activity with aminofluorene as the rapid strain, whereas the activities of other enzymes involved in aromatic amine "activation" are similar (Hien et al., 1986). Additional work (Trinidad et al., 1989) further established that the difference was consistent for a variety of aromatic amines (e.g., p-aminobenzoic acid) but not for nonaromatic amines (e.g., isoniazid).

Glutathione S-Transferase

Glutathione and the glutathione S-transferase (GST) enzymes comprise one of the most important systems in the phase II metabolism or deactivation of active metabolites. The capacity of this system is controlled by the amounts of glutathione available and the activity of the GSTs. As reported by James and Harbison (1982), there is little difference between rats, guinea pigs, and hamsters with regard to the concentration of reduced glutathione, ranging from 6.5 to 9.3 µmol/g liver. They also reported that SKF 525-A as well as other chemicals that form metabolic intermediate complexes with cytochrome P-450 causes decreases in glutathione in the hamster. Stein et al. (1988) reported a broader range, 4.4 (rat) to 10.6 µmol/g (mouse), with a mean for the hamster of 5.4 µmol/g. Igarashi et al. (1986) reported a similar range, 3.5 (guinea pig) to 7.8 µmol/g (mouse), with the mean for hamsters of 5.4 µmol/g. Fasting had no effect on glutathione concentrations in hamsters, but provoked decreases of 47%–65% in the other species examined. Igarashi and coworkers (1986) have also examined GST for species-specific differences. When GST activity was examined using crude cytosol as the enzyme source and 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, hamsters had the highest activity compared to the rat, mouse, guinea pig, and rabbit. The GST of the hamster is made up of three different subunits ranging in weight from 27 to 28.5 Da, as identified by SDS-PAGE (Igarashi et al., 1986). On S-sepharose column chromatography, hamster GST activity eluted as four apparent isozymes (presumably different combinations of subunits).

Rigorous exploration of the substrate specificities of the different hamster isozymes has not been reported. Lam (1988) reported a value for GST activity (7.73 ± 0.46), measured under saturating conditions that was in good agreement with that reported by Igarashi et al. (1986). They also reported that the hamster differs from other species in that subchronic treatment with BHT does not cause an increase in GST. In summary, the hamster possesses an active multizymic cytosolic GST system and adequate amounts of glutathione for this system to a play role in xenobiotic metabolism and toxicity, but it has not been thoroughly explored.

GST is generally considered to be a cytosolic enzyme. As reviewed by Morgenstern et al. (1984), however, the existence of a distinct microsomal GST has been known since the early 1980s. There is sufficient evidence that this is a distinct enzyme and not a contaminant of microsomal preparations by cytosolic remnants. For example, in in vitro assay systems, it is allosterically activated by N-ethylmaleimide, and only with such activation does the specific activity approach that of the cytosolic enzymes with the more traditional substrates, such as CDNB. As reported by Morgenstern et al. (1984), the hamster has the highest specific activity for microsomal GST of any of the common laboratory species. For example, male hamsters have an activity of 957 ± 58 nmol/min/mg (with CDNB in N-ethylmaleimide-stimulated hepatic microsomes), whereas male rats have an activity of 540 ± 25 nmol/min/mg under the same conditions. While on the basis of specific activity and total amounts, the microsomal GST comprises only a small portion of the total hepatic GST, it can still play an important role in the metabolism and toxicity of specific chemicals. For example, in hamsters, hexachloro-1,3-butadiene is preferentially metabolized by the microsomal GST by a ratio of 22.7 to 1.0 over the cytosolic enzyme (Oesch and Wolf, 1989). Similar ratios were 37.7 and 3.3 for human and rat preparations, respectively. The hamster may, therefore, be a better species than the rat in the study of this enzyme.

Conjugation Reactions: Glucuronide, Sulfate, and Amino Acids

Enzyme systems other than GST are involved in phase II reactions. As a loose rule of thumb, these other systems tend to act on more stable chemicals, such as aromatic acetates, phenols, or primary amines, than GST. These other systems catalyze the formation of glucuronic acid, sulfate, and amino acid conjugates. This would also include acetylation, which has been previously discussed. The activity of these systems has been at least partially examined in the hamster. Huckle et al. (1981a) studied the species differences in the in vivo metabolism of 3-phenoxybenzoic acid. Glucuronide conjugates predominated in the hamster, whereas the sulfates predominated in rat. They noted that the hamster, like other rodents and unlike the cat or ferret, forms a very low percentage of amino acid (glycine and taurine) conjugates. In fact, the hamster has the lowest activity of any rodent of glycine N-acyl transferase activity measured in vitro under optimum conditions (Huckle et al., 1981b). Emudianughe et al. (1987a) examined the in vivo metabolism of 1-naphthylacetic acid and observed that the predominant urinary metabolite in hamsters was the glucuronide (64%), whereas the glycine conjugate comprised a smaller percentage (10%). One could generalize that if a functional group could be conjugated with either glucuronic acid or an amino acid, the former will always predominate in the hamster. This, however, is not the case. Emudianughe et al. (1987a,b) compared the metabolism of 1- versus 2-naphthylacetate and found that this chemical was such a poor substrate for uridine-diphospho-glucoronosyl (UDP-glucuronosyl) transferase that even in the hamster amino acid conjugates (glycine and glutamine) were the major metabolites. Hence, depending on the substrate or metabolite available, the hamster will tend to form glucuronide metabolites at the expense of amino acid conjugates, but there are exceptions.

Relatively little work has been done on the UDP-glucuronosyl transferase (a microsomal enzyme) in hamsters since the 1970s. Hietanen and Vainio (1976) compared the in vitro activity of UDP-glucuronosyl transferase in the rat and hamster using p-nitrophenol as the substrate. They observed that on a microsomal protein basis, the hamster had higher activity, but when corrected for the

n-ficrosomal protein and expressed on a gram liver basis, the activity of the two species was about the same (8–12 nmol/min/g). Rats have somewhat higher UDP-glucuronosyl activity with thyroid hormone (T4), a natural substrate, than hamster (Henry and Gasiewicz, 1987), and TCDD treatment induced increases in both species. Interestingly, TCDD provokes completely different changes in circulating thyroid hormone concentrations in rats (decreases) and hamsters (increases), a difference than cannot be accommodated by the difference in UDP-glucuronosyl transferase. Based on the available data, there appears to be quantitative differences in the activity of this enzyme between the rat and the hamster that will require additional work to delineate.

Acetaminophen Metabolism and Toxicity

UDP-Glucuronosyl transferase and 3′-phosphoadenosine-5′-phosphosulfate (PAPS) sulfotransferase (a cytosolic enzyme) frequently act on the same substrates, and the differences in substrate specificity and the availability of cosubstrate (PAPS availability is often limiting) determine which conjugate is preferentially formed. This has been studied in the hamster in the metabolism of acetaminophen. As mentioned, the hamster is among the most sensitive of species to the hepatotoxic effect of acetaminophen, a phenomenon believed to be due, at least partly, to the higher rates of N-hydroxylation by hamsters. This was convincingly demonstrated by Green et al. (1984), who examined the metabolism and toxicity of acetaminophen in hepatocytes isolated from various species. As expected, the hamster produced a higher ratio of toxic to nontoxic (i.e., sulfates and glucuronides) metabolites than other species. This was confirmed by Tee et al. (1987), who demonstrated (1) that hepatocytes isolated from several different species, including the hamster, were equally sensitive to cell injury when exposed to the putative toxic metabolite of acetaminophen, N-acetyl-p-benzoquinoneimine, and (2) hamsters have higher rates of production of this metabolite than other species. Even in hamsters, however, the majority of acetaminophen is disposed of via conjugation to nontoxic metabolites.

As demonstrated by Roberts et al. (1986), sulfate formation is the preferred conjugative pathway of acetaminophen metabolism in isolated hamster hepatocytes. Miller and Jollow (1987) also demonstrated that the sulfate was the preferred conjugate in hamster hepatocytes, especially in incubations fortified with inorganic sulfate. Further, pretreatment of intact hamsters with an inhibitor of PAPS sulfotransferase (2,6-dichlorodinitrophenol; 10 mg/kg ip given 30 min before acetaminophen) greatly enhanced acetaminophen-induced liver injury. In vivo, however, the glucuronide is the preferred conjugate. Miller and Jollow (1987) concluded that this was due to the capacity (as evidenced by the K) of the sulfotransferase, and not the availability of PAPS. Brzenznicka et al. (1987) measured the concentrations of PAPS and observed that the concentration in hamster was about half that in rats (approximately 68 vs 33 nmol/g liver). These observations are not mutually exclusive, and it is likely that both the capacity of sulfotransferase and the availability of PAPS are the reasons that the sulfotransferase does not play a greater role in the metabolism (and, therefore, protection against hepatotoxicity) of acetaminophen in hamsters.

Diethylstilbestrol Metabolism and Toxicity

The sensitivity of hamsters to the hepatotoxicity of acetaminophen is an example of a quantitative species difference in toxicity that has a metabolic explanation. The response of hamsters to DES meanwhile is an example of a qualitative species difference, that is, DES causes kidney tumors in male hamsters, but not in any other species studied. Gottschlich and Metzler (1980) extensively studied the metabolism of DES in hamsters and could not identify any species or sex-related differences in the metabolism of DES to explain the difference in toxicity. This work was continued (Blaich and Metzler, 1988a,b) and has produced some interesting findings. For example, cotreatment of hamsters with 7,8-benzoflavone and DES leads to a reduction in the kidney tumor incidence

and a rise in liver tumor incidence. There is still, however, no evidence that the metabolism of DES plays a role in the induction of renal tumors by this chemical in hamsters. The more rapid hepatic metabolism of DES induced by 7,8-benzoflavone probably plays a role in the protection to the kidney afforded by this treatment regimen.

Extrahepatic Metabolism

Up to this point, this section has focused on hepatic xenobiotic metabolism as the liver is quantitatively the most important organ in xenobiotic transformations. Other organs can also possess metabolic capacity that can play a role in xenobiotic metabolism and toxicity. Those of the hamster will be briefly mentioned here. Burke and Prough (1976) examined the MMFO activity of hamster liver and lung with a variety of different substrates. In general, the lung had much lower activity (e.g., 0.05% for AHH), but for a few substrates, activity in the lung can approach that of the liver (e.g., 77% with biphenyl), and MMFO activity in the hamster lung is essentially unresponsive to induction by 3-methylcholanthrene. Smith et al. (1986) demonstrated that the kidney contains cytochrome P-450 (and the other components of the MMFO) at about 10% (on a microsomal protein basis), and that increases in the activity of the renal MMFO were not induced by common hepatic MMFO-inducing agents. Li et al. (1983) demonstrated that AHH activity of the male hamster kidney is about 20% of that of the kidney and that various antiandrogenic and/or estrogenic treatments will depress the activity of the kidney but not the liver. Wiebkin et al. (1984) reported that isolated hamster pancreatic acinar cells have low (16- to 210-fold less than the rat) but measurable rates of MMFO activity with a variety of different substrates. Pretreatment of hamsters with common inducers had no effect on acinar MMFO activity. Hietanen and Vainio (1976) reported that unlike other species examined, the hamster has no measurable intestinal (duodenum) AHH activity, but does have UDP-glucuronosyl transferase activity (about 25% on a microsomal basis when compared to hepatic activity). Hein et al. (1986) demonstrated that the hamster kidney has higher levels of sulfotransferase than the liver. Kawakubo et al. (1988) reported that hamster skin has a surprisingly high N-acetyl transferase activity that could play a role in carcinogen activation in this species. Hadley and Dahl (1983) reported the MMFO activity of hamster nasal tissue to be higher than any other species examined.

Gut Flora Metabolism

As reviewed by Rowland et al. (1986), the gut flora can also play a role in xenobiotic metabolism and toxicity. The gut flora metabolic activity in rodents tends to be lytic (3-glucosidase and 6-glucuronidase) or reductive (azo, nitro, and nitrite reductases) in nature. The rat and hamster have essentially equivalent activities of 6-glucosidase (30–35 μmol/h/g feces), azo reductase (2–3 μmol/h/g feces), and nitroreductase (3.8–4.2 μmol/h/g feces). The rat has much higher activities with P-glucuronidase (156 vs 60.8 μmol/h/g feces) and nitrate reductase (3.9 vs 1.7 μmol/h/g feces).

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The Guinea Pig

Shayne Cox Gad and John Peckham (deceased)

CONTENTS

TOXICOLOGY

Journalists often refer to human research subjects as "human guinea pigs," and the public mind has long regarded the guinea pig as the classic laboratory animal for all biomedical research and safety assessment. Actually, their use is now proportionately constant at 2% of the annual total of laboratory animals. This makes them only the third or fourth most popular species in toxicology/safety assessment.

Though not used in the numbers perceived by the general public, guinea pigs are important research animals that are used primarily in studies of immunology, audiology, and infectious diseases. They are relatively expensive, usually costing three to five times as much as rats and often

more than rabbits; however, perhaps their greatest disadvantage for research use is the lack of readily accessible peripheral veins for intravenous injections and collection of blood and serum samples. As will be seen, however, they are more widely used in safety assessment than most believe.

Species and Characteristics

The scientific name of the guinea pig is *Cavia porcellus*. Guinea pigs are hystricomorph rodents (suborder Hystricomorpha, order Rodentia) originating from South America. They are more closely related to porcupines and chinchillas than to mice or rats.

Guinea pigs have long been used as experimental animals in biomedical research because they are small, tame, and easy to handle. Of the three natural varieties (English, Abyssinian, and Peruvian), the albino form of the short-haired English variety is the most commonly utilized in the laboratory. The Hartley strain of this variety is by far the most commonly used in toxicology. Recently, the Charles River Laboratories has also developed a fertile, euthymic hairless strain that offers advantages for use in dermal studies (Alden, 1985).

The popularity of the guinea pig as a pet and research animal owes much to their docile nature. They seldom bite or scratch and will respond to attention with frequent and gentle handling. At the same time, they tend to be messy in their habits, and the development of human allergies to guinea pigs is not uncommon (in the toxicology laboratory, probably only allergies to the rabbit and rat are more common).

In many ways, this roly-poly rodent is the most attractive laboratory animal. Exceptionally curious, the guinea pig investigates any activities outside of its cage by attempting to watch and expressing itself by a wide range of sounds from a deep chortle to a shrilling whistle. They are unable to climb, but they can jump short distances.

Guinea pigs are alert and full bodied and have smooth, shiny skin. Their coat is dense, with the hair being clean and not marked by discharge from the nose, eyes, or ears. If allowed to feed freely, guinea pigs can become quite large over time. Males will commonly reach 1 kg at 1 year and can weigh several kilograms at a later age.

History

The guinea pig was first scientifically described by Gesner (1516–1605) and Aldrovandus (1522–1607) in about 1580 (Wagner, 1979). The use of the guinea pig as a test animal for biomedical research goes at least back to Lavoisier in 1780 (Lane-Petter, 1963), who used it to measure heat production by animals.

In 1979, Collins reported that (for 1965) about 2.5 million guinea pigs were used annually for research in the United States. Over more than 20 years since, this figure has undoubtedly decreased. By 1983, the total use of guinea pigs in research was 521,237 (of which 28,753 were used in toxicology).

Utilization as a Test Animal

In the broad range of biomedical research, the guinea pig has been employed as the test animal in a wide range of investigations: nutrition, pharmacology, allergy, radiology, and immunology. Complement, a substance originally isolated from the blood of guinea pigs, is a key component in serological work.

The guinea pig can be easily infected by human tuberculosis germs and is indeed the animal of choice for the diagnosis of tuberculosis in humans. Another disease that the guinea pig shares with its fellow mammal, the human, is scurvy. This disease is noninfectious, being caused by a deficiency of vitamin C in the diet. An idle but interesting fact is that the only other animal subject to scurvy, the monkey, is also very susceptible to human-type tuberculosis.

Table 5.1   Toxicological End Points Evaluated Using the Guinea Pig as a Model

End Point	References
Delayed contact dermal sensitization (Coombs type IV) Buehler, Draize, Landsteiner, guinea pig maximization, etc., test designs	Landsteiner and Chase (1937, 1940, 1941, 1942) Draize et al. (1944), Buehler (1964), Muraoka et al. (1968), Magnusson and Kligman (1969), McMichael et al. (1983), Christensen et al. (1984), Martin and Maibach (1988)
Immediate hypersensitization	Ayala et al. (1988)
Photosensitization	Ichikawa et al. (1981), Harber and Shalita (1975)
Armstrong and Harber and Shalita tests	
Ototoxicity (because of early findings with antibiotics and nonsteroidal anti-inflammatory drugs)	Brummett (1983), Cazals and Guilhaume (1985), Parravicini et al. (1983)
Cataractogenesis (induction of cataracts by dermal and/or oral application of test substances)	Melnikova and Radionov (1979), Lechat et al. (1984), Lunam et al. (1985), Kapusnik and Sande (1986), Mackic et al. (1994), Nomura et al. (2001)
Pulmonary irritation and sensitization	Emerson and Cole (1983), Karol et al. (1980)
Systemic toxicity (in place of traditional species such as rat, mouse, and dog). Usually due to efficacy data being developed in carrageenan colitis model in this species, aminoglycoside toxicity	Adrian et al. (1976), Lee and Thomsen (1982)
Screening for adverse cardiovascular effects, particularly in a Langendorff or other in vitro preparation	Guo et al. (2009), Kagstrom et al. (2007), Fossa et al. (2007)
Host resistance assay (for generalized screen of effect on immune competence, in place of traditional mouse model in same assay)	McFarland et al. (2008)
Carcinogenicity	Hottendorf (1985)
Teratology	Hoar (1969), Rocca and Wehner (2009)
Inhalation	Firpo et al. (1988)

In safety assessment/toxicology studies, however, the use of the guinea pig is a bit narrower. Table 5.1 summarizes these uses. Specific protocols for some of these tests will be discussed later in this chapter.

Basic Biological Characteristics

During the course of all the different kinds of toxicology studies that need to be designed and conducted, a complete knowledge of the fundamental biological characteristics is essential. Also, most studies are conducted with the measurement of various parameters to determine if a test material has had any (particularly any adverse) effect on the animals. Such measurements must start from a position of knowledge of what normal values are and of what kind of variability is commonly seen.

Tables 5.2 through 5.9 summarize a range of basic biological characteristics of the guinea pig. Most of these were gained from the review of general sources (e.g., Collins, 1979), though a number of special references are cited in the tables. The tables summarize physiological (Table 5.2), reproductive (Table 5.3), hematology (Table 5.4), clinical chemistry (Table 5.5), serum enzyme (Table 5.6), serum protein component (Table 5.7), organ weight (Table 5.8), and deoxyribonucleic acid (DNA) repair (Table 5.9) characteristics (Melby and Altman, 1976; Mitruka et al. 1976; Holmes, 1984; Burns et al., 2001; Zuokiga-Gonzalez et al., 2001). Figure 5.1 shows growth curves for male and female animals.

Husbandry

Guinea pigs represent an intermediate species in terms of ease and expanse of care. While they are considerably easier to house and maintain than primates, dogs, or rabbits, they are clearly more expensive and difficult than rats or mice (Ediger, 1976; Hutchinson, 1983).

Table 5.2   Physiological Parameters

Life span	2–6 years	
Rectal temperature	38.6°C–40.0°C	
Respiration rate	69–160/min	
Heart rate	240–277/min (Fara and Catlett, 1971) or 130–190/min (Collins, 1979)	
Daily food consumption	8 g/100 g body weight	
Daily water consumption	10 mL/100 g body weight	
Percent of total life span	Embryonic	1.8%
	Gestational	4.4%
	Puberty	4.2%
Oxygen consumption	0.76 mL O_2/g/h	
Tidal volume	1.8 mL	
Minute volume	0.16 L	
Mean blood pressure (mm Hg)	Systolic	76.7
	Diastolic	46.8
Metabolic rates (resting)	3–5 weeks of age	34.0 cal/m²/h
	7–9 weeks of age	33.25
	11–13 weeks of age	32.95
	5–6 months	30.8
	11–12 months	29.54

Table 5.3   Reproductive Parameters

Puberty	45–70 days
Breeding age	12–14 weeks
Gestation	59–70 days; 63 average
Weaning age	21–28 days
Diploid chromosome number	64
Litter size	1–8; 3–4 average
Estrus cycle	16–18 days

The objective of all that is done for the husbandry of laboratory animals can be distilled to maintaining healthy animals in as stable an environment as possible and as humanely as possible.

One set of broad principles that should be taken into account when assessing whether animals are being humanely maintained and utilized are the so-called five freedoms (Webster, 1986). These are the following:

1. Freedom from thirst, hunger, and malnutrition—achieved by readily accessible fresh water and diet to maintain full health and vigor
2. Appropriate comfort and shelter
3. Freedom from injury and disease—achieved by prevention or rapid diagnosis and treatment
4. Freedom of movement and the opportunity to express most normal patterns of behavior
5. Freedom from fear

Though in toxicological research some of these five principles are not strictly possible, to the extent possible they should guide the researcher's actions.

Housing and Caging

Eveleigh (1988) has recently reviewed the history of the development of caging for several laboratory species, including the guinea pig, and the interested reader is directed to that source.

Table 5.4   Hematology Parameters

Plasma volume	30.6–38.2 mL/kg body weight	
Whole blood volume	75 mL/kg body weight	
RBC diameter	7.1 μm	
RBC sedimentation rate	0.5–1.5 mm/h	
Blood pH	7.35	
RBC	$(4.5–7.0) \times 10^6/mm^3$	
Micronuclei	0.2 ± 0.4 (1–5 days old)	
	0.3 ± 2.8 (adults) (Zuniga-Gonzalez et al., 2001)	
Hematocrit	42.0–55.2 mL/100 mL	
Platelets	$(3.4–10.0) \times 10^5/mm^3$	
Hemoglobin	11.7–14.5/100 mL	
WBC	$(9.9–10) \times 10^3/mm^3$	
Differential	Neutrophils	$4.2 \times 10^3/mm^3$
	Eosinophils	0.4
	Basophils	0.07
	Lymphocytes	4.9
	Monophils	0.43
PVC (%)	43	
MCV (cuμ)	81	
MCH (μμg)	25	
MCHC (%)	30	
Reticulocytes (%)	0.9–1.0	
Red blood cell	Diameter	7.1 μm (Scarborough, 1931; Ponder, 1948)
	Life span	60–80 days (Edmondson and Woburn, 1963)

Table 5.5   Clinical Chemistry Parameters

Plasma	Calcium	5.3 mEq/L
	Sodium	145–152 mEq/L
	Chloride	105 mEq/L
	Phosphorus	5.3 mg/100 mL
	Potassium	6.8–8.9 mEq/L
	Magnesium	2.3 mg/100 mL
	Cholesterol	21–43 mg/100 mL
	Serum protein	5.4 g/100 mL
	Albumin	2.8–3.9 g/100 mL
	Globulin	1.7–2.6 g/100 mL
	Protein-bound iodine	1.8–2.2 mμg/100 mL
Whole Blood	Glucose	82–107 mg/100 mL
	Bilirubin	22–39 mg/100 mL
	Non-protein-bound nitrogen	30–51 mg/100 mL
	Creatinine	0.99–1.77 mg/100 mL
	Blood urea nitrogen	30–51 mg/100 mL
	Glucose	79–107 mg/100 mL
	Uric acid	1.3–5.6 mg/100 mL
	Total lipids	94–245 mg/100 mL
	Phospholipids	25–77 mg/100 mL
	Triglycerides	0–145 mg/100 mL
	Progesterone	0–2.75 ng/100 mL
	Estrogen	0–54 pg/100 mL

Table 5.6   Serum Enzyme Activities

| | Values | | | |
| | Male | | Female | |
Enzyme	Mean	SD	Mean	SD
Amylase (Somogyi units/dL)	295.0	31.0	269.0	28.0
Alkaline phosphatase (IU/L)	74.2	6.92	65.8	5.46
Acid phosphate (IU/L)	32.2	2.59	28.7	3.20
Alanine transaminase (SGPT) (IU/L)	44.6	6.75	38.8	7.15
Aspartate transaminase (SGOT) (IU/L)	48.2	9.5	45.5	7.00
Creatine phosphokinase (CPK) (IU/L)	0.95	0.15	1.10	0.20
Lactic dehydrogenase (LDH) (IU/L)	46.9	9.50	52.1	11.2

Sources: Data from Albritton, E. C., *Standard Values in Blood*, W.B. Saunders Company, Philadelphia, PA, 1952; Altman, P. L. and Dittmer, D. S., *Biology Data Book*, FASEB, Washington, DC, 1964; Altman, P. L. and Dittmer, D. S., *Respiration and Circulation*, FASEB, Bethesda, MD, 1971.

Table 5.7   Serum Protein Components of the Normal Guinea Pig

| | Values | | | |
| | Male | | Female | |
Fractions	Mean	SD	Mean	SD
Total protein (g/dL)	5.6	0.28	4.80	0.34
Albumin (g/dL)	2.73	0.30	2.42	0.27
(%)	48.8	5.50	50.5	5.40
α_1-Globulin (g/dL)	0.11	0.04	0.10	0.02
(%)	1.90	0.38	2.20	0.19
α_2-Globulin (g/dL)	0.33	0.08	0.23	0.06
(%)	5.90	1.25	4.80	1.42
β-Globulin (g/dL)	1.14	0.20	0.82	0.17
(%)	20.4	4.10	17.1	3.60
γ-Globulin	1.29	0.26	1.22	0.15
(%)	23.1	4.60	25.4	3.25
Albumin/globulin	0.95	0.16	1.02	0.18

Sources: Data from Spector, W. S., *Handbook of Biological Data*, W.B. Saunders Company, Philadelphia, PA, 1961; Burns, K. F. and De Lannoy, C. W., *Toxicol. Appl. Pharmacol*., 8, 429, 1966; Altman, P. L. and Dittmer, D. S., *Biology Data Book*, 2nd edn, FASEB, Bethesda, MD, 1974; Dimopoullos, G. T., Plasma proteins, in *Clinical Biochemistry of Domestic Animals*, Cornelius, C. E. and Kaneko, J. J., eds., Academic Press, New York, 1963, pp. 109–201; Moreland, T. A., *Biochem Pharmocol*., 24, 1953, 1974.

Since guinea pigs do not climb, they can be kept in open-sided boxes or pens, provided that the sides are at least 10 in. high.

Guinea pigs may be housed indoors in pens on the floor, in fixed or portable tiered compartments, or in cages. Floor pens, despite their disadvantages (such as waste of space and spread of infection), are commonly used because of the simplicity and ease of cleaning and inspection. The general size of a pen is 40 in. × 96 in., and it can be placed on each side of the room, leaving a passage of about 24 in. wide. Another arrangement uses only one-half of the available space each day.

Tiered compartments may also be used. These are permanent compartments made of concrete or wooden shelves or portable metal units. The permanent type is not much more economical in

Table 5.8 Organ Weights in Two Strains of Guinea Pigs[a]

	Weight (g) ± SD										
	Strain 2			Strain 2			Strain 13			Strain 13		
	Male			Female			Male			Female		
Organ												
Body	802	±	65	780	±	69	1044	±	69	940	±	99
Liver	25.37	±	2.9	29.21	±	4.83	33.7	±	3.89	35.5	±	8.50
Lungs[b]	5.21	±	0.97	5.18	±	1.22	7.23	±	0.94	7.44	±	0.88
Heart	2.12	±	0.28	2.07	±	0.27	2.42	±	0.33	2.26	±	0.23
Thyroid[c]	0.061	±	0.013	0.058	±	0.007	0.078	±	0.017	0.074	±	0.924
Kidney[c]	2.94	±	0.56	2.79	±	0.38	2.57	±	0.24	2.33	±	0.15
Adrenal[c]	0.402	±	0.13	0.394	±	0.110	0.310	±	0.050	0.284	±	0.041
Spleen	0.78	±	0.12	1.03	±	0.27	0.73	±	0.07	0.93	±	0.16

Source: Breazile, J. E. and Brown, E. M. Anatomy, in: *The Biology of the Guinea Pig*, Wagner, S. E. and Manning, P. J., eds, Academic Press, New York, 1979, p. 115.

[a] N = 20 in all cases.
[b] Weight of both lungs.
[c] Weight of left organ only.

Table 5.9 DNA Repair Activity (erg/mm^2 UV Light Equiv.)

IQ	9.4
MCIQ	1.7
2-AF	14.2
IQ/2-AF	5.5
MeIQ/2-AF	0.7

Source: Loury, D. J. and Byard, J. L., *Environ. Mutagen.*, 7, 245, 1985.
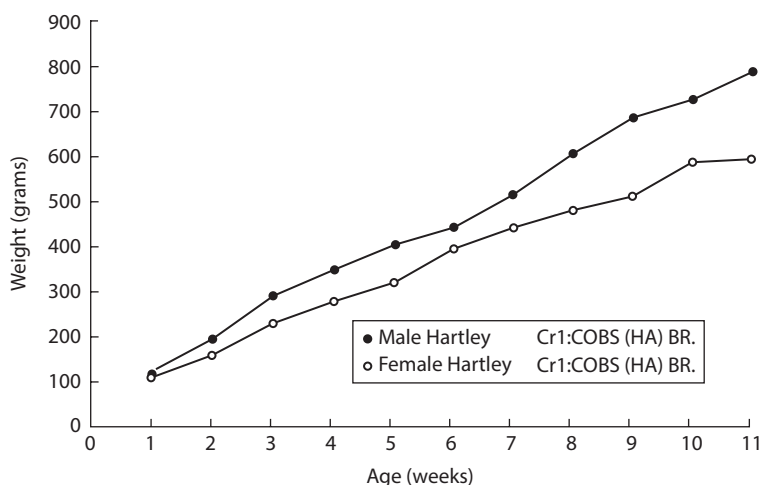
Figure 5.1 Guinea pig weight curves.

space than the floor pens, and they are generally more expensive and difficult to clean and disinfect. The portable type, up to four tiers, are space saving and easy to clean.

The initial cost of a caging system is greater than boxes, pens, or tiered compartments, but it is space saving. Generally, 30 in. × 30 in. × 14 in. cages made out of a single aluminum alloy sheet are ideal. These cages can be divided by panels if needed. Each cage can hold a harem of four sows

and one boar. The cages will hold 12 young animals up to 350 g in weight, and the number may be reduced to 10, 8, and 6 as the weight increases. The cages for experimental animals are 10 in. × 10 in. × 10 in. and are made up of 3/4 in. wire mesh to hold individual animals, and a 24 in. × 16 in. floor area may be enough for 10 guinea pigs. The most common bedding material is seasoned soft-wood shavings. Peat moss, though more expensive, is highly recommended. Treated flax and corn-cobs are also good, as they have highly absorbent and deodorant properties. Cereal straw should not be used because it may be irritating to the animals and may act as a source of contamination.

The Institute of Laboratory Animal Resources guidelines as to floor space to be provided per animal did not change with the 1985 revision of the Guide for the Care and Use of Laboratory Animals. Caging or housing of whatever form should provide (as a minimum) the following space for each animal (NIH, 1985).

	Floor Area	
Weight (g)	per Animal (cm²)	Height (cm)
Up to 250	277 (43 in.)	17.8 (7 in.)
250–350	374 (58 in.)	17.8 (7 in.)
Over 350	652 (101 in.)	17.8 (7 in.)

Attention to the details of thorough cleaning is an essential component of the husbandry of guinea pigs. Obnoxious odors, the accumulation of mineral scale from the high concentration of mineral salts in guinea pig urine, and the spread of infectious diseases between animals and cages are minimized with proper sanitation of cages and racks.

Cages in which bedding is used should be cleaned and sanitized often enough to prevent an accumulation of excreta or debris. This generally means that cages should be sanitized at least once each week, and all new bedding should be added each time the cage is sanitized. Although wire floor cages do not have to be cleaned as frequently, they should be sanitized at least once every 2 weeks. The pan under the cage, however, must be cleaned frequently to prevent the accumulation of excreta. If large numbers of animals are maintained in each cage, daily cleaning of the pans may be necessary to maintain a high level of sanitation. Soiled bedding should be disposed of promptly either by incineration or in a way that will not contaminate other animal areas or areas of human habitation.

Temperature, Ventilation, and Humidity Control

Strict attention should be given to air-handling systems to control temperature, humidity, air velocity, and air pressure within the animal room. Guinea pigs are extremely susceptible to respiratory disease, and well-designed, efficiently operating system provides the essential environmental stability for respiratory disease control.

There should be 10–15 changes of air per hour and no air should be recirculated unless it has been filtered to remove airborne contaminants. Care must be taken to control the velocity and direction of airflow to produce a draftless and even distribution of air to all areas of a room. An ideal state of mass air displacement is, in some instances, difficult to achieve in a room filled with animals and cages, and in these circumstances, cages can be designed or arranged to minimize drafts.

Ideally, room temperatures should be maintained between 65°F and 75°F (20°C–25°C) and relative humidity at approximately 45%–55%.

Breeding

It is general practice now to purchase guinea pigs for toxicological research from specialized venders, and generally, it is not cost effective to try to raise stocks for use in tests. Multiple strains have

been developed (Veterinary Resources, 1981), but the Hartley albino is most common in toxicology. However, there are specialized cases (teratology and reproductive toxicity studies, for example) where successful breeding is an essential component of the test. In such cases, guinea pigs are best mated when they are approximately 3 months old or weight from 450 to 600 g (through "time-mated" animals are available from vendors). Specific considerations of experimental design will generally dictate the details of arrangements for mating, but it should be kept in mind that the reproductive potential of the guinea pig is high and that most simple pair cohabitations result in pregnancy.

Mating in the guinea pig is normally detected by either the presence of sperm in the vagina or the presence of a vaginal plug. The plug consists of a central core formed by a mixture of secretions from the male's vesicular and coagulating glands and is enclosed by a mass of flat epithelial cells apparently derived from the vaginal wall (Stockard and Papanicolaou, 1919). The plug usually fills the vagina from cervix to vulva. A few hours after its formation, the plug falls out of the vagina and can often be observed as a waxy mass on the cage floor. The efficiency of utilizing plugs to predict pregnancy is usually high. Live sperm, on the other hand, can be found routinely in vaginal smears for only a few hours after copulation.

Watering

Most facilities supply water to guinea pigs either from suspended water bottles or from an automatic system through sipper tubes. If bottles are used, it is frequently the practice to supply ascorbic acid in the water. Whichever of these supply methods is utilized, however, the use of either distilled or deionized water rather than chlorinated tap water is recommended as it will reduce the endogenous rate of vitamin C deactivation. Guinea pigs do have a tendency to play with water tubes, which can result in the flooding of cages.

Nutrition

Among the commonly used laboratory species, only guinea pigs and primates require a dietary source of vitamin C (ascorbic acid). Collins and Elvehjem (1958) reported that the ascorbic acid requirement for the growth of immature guinea pigs is 0.5 mg/100 g body weight per day. Nungester and Ames (1948) indicated that a 300 g guinea pig requires a daily intake of approximately 6 mg of vitamin C to provide adequate protection against infection. Under intensive breeding conditions, the requirement of the adult female that is pregnant or lactating during most of her breeding life is at least 20 mg/day (Bruce and Parker, 1947).

Commercial pelleted diets are manufactured to meet the requirements of the guinea pig for ascorbic acid (Anonymous, 1987). Additionally, they are usually fortified to compensate for ascorbic acid losses in storage, but careful attention must be paid to manufacturing dates on such food. Laboratory animal feeds must not be maintained too long in storage and must be fresh when used. Ideally, guinea pig feeds should be stored in rooms where the temperature is 50°F or less and should not be stored in the animal room. The date of manufacture is commonly given or coded on each feed bag, and a strong effort should be made to store feed for no longer than 4–6 weeks from the date of manufacture. This is especially important where no green vegetable supplement is offered. Feeders should be designed to prevent guinea pigs from climbing into them. For this purpose the standard J-type feeder works very well.

Feeding guinea pigs green vegetables in addition to the pelleted diet has been a subject of continuing controversy. A "green" supplement on a regular basis may provide insurance against vitamin C deficiency; however, such supplements offer benefits beyond this one advantage. These benefits are most apparent in a production colony under an intensive breeding system where there is heavy stress on the pregnant or lactating female. Such supplements enhance the ability of breeding animals to maintain body weight. Based on the results of feeding experiments in which four different commercially prepared diets were compared using large numbers of animals held for breeding periods

of 18–24 months. It has been observed that the feeding of a green supplement resulted in an average increase of 10%–44% in weaned offspring over the same pelleted feeds without a green supplement.

Guinea pigs do have some rather specific requirements for amino acids and vitamins in their diets (Navia and Hunt, 1976). Besides vitamin C, they are also very susceptible to vitamin E deficiencies and diets with 34%–33% protein are recommended.

Diseases

Scurvy

Signs of scurvy usually appear within 2 weeks after guinea pigs are deprived of vitamin C. The guinea pig diet should provide 15–20 mg vitamin C/kg body weight for maintenance (Clark and Baker, 1975); vitamin C deficiencies frequently occur when guinea pigs are fed rabbit chow. Although rabbit pellets resemble guinea pig pellets in appearance, rabbit pellets lack vitamin C, are lower in protein, and therefore do not meet the dietary requirements of guinea pigs.

Clinical signs of a vitamin C deficiency are reluctance to move, an unkempt appearance, swellings around the joints, diarrhea, and cutaneous sores. Animals often succumb to secondary bacterial infections before the classic signs of scurvy are evident. The most prominent gross lesions seen at necropsy are hemorrhages in the muscle and periosteum, particularly around the stifle joint and rib cage, and epiphyseal enlargement, best noted at the costochondral junction.

Vitamin E Deficiency

Guinea pigs are very susceptible to vitamin E deficiencies, usually resulting in the development of skeletal muscle necrosis, but a deficiency is unlikely in animals fed commercially produced diets (Wagner, 1979).

Alopecia

A diffuse loss of hair over the flanks and back develops to a degree in all sows in late pregnancy. It is more marked with an intensive breeding program. Around the time of weaning, a thinning of the hair occurs during the transition from baby fur to more mature hair (Wagner, 1976). Alopecia also seems to be associated with other stress conditions, although the exact cause is not known. Nutritional and genetic factors are probably involved to some degree.

Loss of hair with a distinctive pattern or patchy distribution can result from a hair-chewing vice termed barbering. Animals may chew their own hair or that of a cage mate. The location of hair loss can usually provide a clue as to whether it is self-inflicted or has resulted from barbering by a cage mate.

Circumanal Sebaceous Accumulations

Excessive accumulations of sebaceous secretions occur in the folds of the circumanal and genital region in adult male guinea pigs. These folds must be cleansed periodically to preclude infection and unpleasant odors. At times, the sebaceous secretions form a plug that accumulates in the folds between the two halves of the scrotum. It can be removed after softening with soap and water (Williams, 1976).

Preputial Infection and Vaginitis

Male guinea pigs occasionally develop preputial infections caused by lodging of foreign material in the preputial folds. Breeding males on bedding may be affected when pieces of bedding adhere to

the moist prepuce following copulation and are drawn into the preputial fornix. Treatment primarily involves removing the particle and cleansing the area. Genital herpes is also seen (Hsiung et al., 1984).

Vaginitis in female guinea pigs is usually caused by entrapment of wood chips or their bedding in the vagina, causing a foreign body reaction. The problem is corrected by washing the area carefully and wiping away the chips. It may be desirable to place the animal on a different type of bedding until the area has healed.

Care must be taken in using antibiotics – the guinea pig is very sensitive to acute toxicity from beta-lactam (Boyd and Fulford, 1961). Also readily susceptible to parasitic infestations such as ringworm (which is easily transmissable to humans; Pombier and Kim, 1975).

Water Deprivation

Sometimes animals die of water deprivation even when ample water seems to be available. This may occur when (1) water is provided by a device with which the animal is not familiar or does not know how to operate, (2) water devices are placed too high or are otherwise inaccessible, particularly for small weanlings, (3) water is impotable because of impurities or odors in the container or the water itself, or (4) automatic watering systems become plugged or jammed.

Table 5.10 provides an overview of the major infective and parasitic diseases seen in laboratory guinea pigs. Guinea pigs are highly susceptible to factors in the environment that lower their resistance to infection. Among these are poor sanitation, overcrowding, mixing of species, improper temperature and humidity control, inadequate diet (especially insufficient vitamin C), transportation, and experimental procedures (Bivin et al., 1969).

Dosing Techniques

Before any dose may be administered, an animal must first be picked up and manipulated. Guinea pigs should be lifted by grasping the trunk with one hand while supporting the rear quarters with the other. Support is particularly important with adults and pregnant animals. Injured lungs may result if an animal is grasped too firmly over its back.

Oral Dosing (Gavage)

Guinea pigs can be stomach tubed using a technique similar to that for the rat. An assistant restrains the animal by grasping it around the shoulders and supporting the hindquarters to prevent undue struggling. A blunted 15–16-gauge hypodermic needle, polyethylene catheter (3–4 French), or commercially manufactured dosing needle ("Popper tube") as used for rats is introduced into the mouth through the interdental space and advanced gently into the esophagus. A small gag made from a solid plastic rod with a hole drilled centrally may be used to prevent the animal from biting a plastic catheter.

Subcutaneous Injection

The animal should be restrained by an assistant as described earlier and a small area of skin on the flank tented by the operator. The needle is introduced into the raised skin, parallel to the body wall. The skin of the guinea pig is thicker than in smaller rodents and provides more resistance to needle passage, hence injection is easier if a short (0.5–1.0 in.) 21–23-gauge needle is used.

Intradermal Injection

Intradermal injection is carried out as described for rats. The thicker skin of the guinea pig makes the technique relatively easy in this species. As described previously, the presence of a small bleb of material indicates successful intradermal rather than subcutaneous injection.

Table 5.10   Some Common Infectious and Parasitic Diseases of the Guinea Pig

Name	Pathogenic Agents	Symptoms—Appearing Singly or in Various Combinations	Gross Lesions
Virus pneumonia	Virus Latent—no change	Acute—unthrifty, emaciated	Lungs
Pseudotuberculosis	Bacterial—*Y.* or *Pasteurella pseudotuberculosis*	Acute—rapid breathing, rales, diarrhea, enlarged lymph nodes, emaciation, unthrifty Latent—no change (organisms may be recovered from throat)	Lungs, liver, and spleen show whitish, caseous abscesses
Bacterial pneumonia	*Bordetella bronchiseptica* and *Streptococcus pneumoniae*	Acute—rales, discharge from nose, enlarged lymph nodes Latent—no change (organisms may be recovered from throat)	Lungs
Lymphadenitis	Bacterial—*Streptococcus pyogenes* Group C	Acute—gross swelling of lymph nodes in neck region; unthrifty, emaciation Latent—no change (organisms may be recovered from throat)	Lymph nodes, pericardium
Salmonellosis	Bacterial—*S. typhimurium*, *S. enteritidis*	Acute—slight diarrhea, unthrifty, emaciation Latent—no change (organisms may be recovered from feces)	Enlargement of gallbladder and liver
Wasting disease	Unknown (probably a virus)	Acute—inappetence, rapid loss of weight, unthrifty Latent—no change	None
Salivary gland virus	Cytomegalovirus	Acute—large eosinophilic intranuclear inclusion of the salivary gland ductal epithelium, loss of appetite Latent—none	Swollen glands

Scientific Name	Common Name	No. of Legs (May Be Rudimentary)	Food	Remarks
G. ovalis	Louse	6	Blood	Adults visible to naked eye
G. porcelli	Louse	6	Blood	Adults visible to naked eye

Scientific Name	Common Name	Shape	Host Tissue Invaded	Remarks
Paraspidodera unicata	Round worms	Long, narrow smooth body	Intestines	Eggs may be seen (microscopically) in feces. Adults may be seen by naked eye in feces.
Two species 1. *E. caviae* 2. *Balantidium caviae*	Coccidia	Spherical	Intestines	Eggs (oocysts) may be seen (microscopically) in feces.

Footpad Injection

The footpads are occasionally used as injection sites, particularly of material intended to act as an antigen for antisera preparation. The animal should be restrained by an assistant as described earlier and injection made into the large central pad of the foot. In view of the considerable swelling that often ensues, only one pad should be inoculated, so that the animal can avoid placing full weight on that limb. Since there is little evidence to suggest that footpad inoculation of antigen results in any better antibody production than does inoculation at other sites, it is preferable to avoid using this technique.

Intraperitoneal Injection

A similar technique to that described in the rat and the mouse is used for intraperitoneal injection of guinea pigs. The animal should be restrained by a second individual. The operator extends one of the animal's legs and introduces the needle along the line of the thigh into the center of the posterior quadrant of the abdomen.

Dermal Application

Either in vivo (as in sensitization studies) or in vitro (on patches of skin mounted in a Franz cell—Barbero and Frasch, 2009), the guinea pig sees its most significant utilization via this route. The back of the animal is shaved and materials are applied the following day directly to the shave area. Occlusive patching over the application site is frequently used. The skin permeability is better than the rat and comparable to rabbits and humans (Bartek, 1972).

Intramuscular Injection

As with other rodents, the anterior and posterior thigh are the sites most frequently used for intramuscular injection, although it is also possible to inject into the triceps muscles on the anterior aspect of the shoulder. To inject into the thigh, the animal should be restrained by an assistant as for intraperitoneal injection and one leg held firmly by the operator. If the quadriceps muscles are to be used, they should be held between the thumb and forefinger, and the needle introduced at right angles to the skin into the center of the muscle mass.

Intravenous Injection

Guinea pigs have few superficial veins; those which are reasonably accessible are the ear veins and the penile vein (in males). These veins are small and fragile, and hence, intravenous injection is difficult in this species. In large (>500 g) guinea pigs, the ear veins should be used. The ear should first be swabbed with a small quantity of xylene to dilate the vessels, and the animal restrained by an assistant on a firm surface.

The ear should be held firmly at one edge, and a suitable vein selected. A very fine needle (29–30-gauge) should be used, and once positioned in the vein, the syringe can be steadied using the remaining fingers of the hand restraining the ear. Any movement of the animal during this procedure is likely to result in damage to the vein, and to avoid this, it may be preferable to anesthetize the animal. Following successful venipuncture, the xylene should be removed from the ear using a wet swab.

Penile vein injections should always be carried out in anesthetized animals, since the procedure may cause considerable discomfort. The penis is extruded from beneath the inguinal skin by pressure at either side of the genital opening. The tip should then be grasped between the thumb and forefinger and the organ extended and rotated so that the dorsal penile vein lies uppermost. When introducing the needle, care should be taken to avoid damaging the fragile vein.

A second approach is to use the saphenous vein or the dorsolateral vein of the penis. A cutdown may be required for exposure of the saphenous vein. Guinea pig skin is tough, especially over the back, and subcutaneous injections require a degree of force.

Common Techniques

Blood Collection and Measuring Blood Flow and Blood Pressure

Obtaining blood samples from guinea pigs is almost as difficult as intravenous injections. Methods commonly employed include cutting the nail bed; puncture of the dorsal metatarsal vein

of the marginal ear vein; puncture of the orbital sinus; vacuum-assisted bleeding of either the lateral marginal vein of the hind limb, or the lateral metatarsal vein, cardiac puncture; or via an indwelling cannula technique. Carbon dioxide anesthesia increases the yields of blood and serum particularly when exsanguinating the animal. A method of obtaining 3–6 mL of blood from the femoral artery has been described by Simpson et al. (1967), and Burnett et al. (1968) and Hem et al. (1998) have developed a method of collections from the saphenous vein that yields good samples.

Blood flow through the spleen has been determined with xenon-133, based on the measurement of the rate of the disappearance of xenon-133 from the spleen. Similarly, uterine blood flow was measured near term, and a technique for recording the blood pressure of fetal guinea pigs was described briefly by Davitaya and Nadirashvili (1971).

Guinea pigs lack readily accessible peripheral veins, having deeply placed vessels often covered by layers of fat. A small amount of blood can be collected from the orbital sinus or by clipping a toenail. Larger amounts of blood can be obtained from the femoral artery or vein or directly from the heart. Cardiac puncture is best carried out with the animal lightly anesthetized. The guinea pig can be placed either on its right side and the needle inserted at the site of the apex beat around the fourth intercostal space or on its back and the needle inserted in the xiphoid area, aiming for the left ventricle. Cardiac punctures in the guinea pig, however, involve a significant element of risk.

Shaving and Tape Stripping

Two main techniques are common to most dermal toxicity studies performed in the guinea pig. These are shaving (for the removal of hair from an area that it is desired to apply test material to) and tape stripping (for the removal of the stratum corneum, or barrier layer, of the skin).

Shaving

The Oster Model AS electric clippers with detachable blades are most commonly used to shave guinea pigs, rabbits, and rodents:

1. The guinea pig is restrained manually with sufficient downward pressure on the anterior (neck and shoulder) area and posterior (haunches and rump) area of the animal.
2. Initially, the Oster Model AS electric clipper with blade size 10 is used. Size 10 cuts the hair to a length of approximately 1/16 in.
3. The clipper blade is held parallel to the animal's skin. Proceeding slowly, the hair is shaved against the grain of hair growth, giving the blade a chance to feed and cut. The skin of the animal is stretched to remove wrinkling, which both facilitates shaving and reduces the risk of cutting or tearing the skin.
4. An assistant, helping to restrain the animal with one hand, uses the other hand to guide the nozzle of a vacuum cleaner in front of the electric clipper to remove hair clippings during the shaving process. The hair that is collected in the vacuum is emptied into a plastic bag and disposed of as waste.
5. After the hair of the back and both flanks of the guinea pig have been clipped to a length of 1/16 in., the size 10 blade is removed and a size 40 blade is attached to cut the remaining hair to a length of approximately 1/130 in. following steps 3 and 4 earlier.
6. The blades should be changed periodically to reduce overheating and resultant skin irritation.

Each guinea pig is examined for any signs of abrasion/irritation that may have occurred during shaving. If abrasion is present, this should be recorded.

Blade Maintenance

Proper maintenance of cutting blades is essential. Cutting blades used in one toxicity study should never be used in another toxicity study unless they are first cleaned thoroughly with an

appropriate solvent, such as acetone or ethanol. Once the blades are cleaned with the solvent and oiled with clipper oil, they may be used in a different toxicity study. If several toxicity studies are performed simultaneously, it is important to use a different blade set for each individual study in order to prevent cross-contamination. One blade set may be identified for shaving all control animal groups of different studies.

Cleaning Blades — Should cutting blades fail to cut, examine the cutting surfaces of the upper and lower cutting blade units. If hair or foreign matter is present, remove it; blades will not cut when hair or foreign matter is present. Brush the blades with the small brush supplied with the clippers to remove the hair and/or foreign matter buildup.

If more extensive cleaning is needed, the cutting blades should be detached from the clipper. Without separating, slide the upper blade approximately half way to either side. (Do not remove tension spring fastened to lower blade by two screws; doing so will disturb the cutting tension.) New blades or very dirty blades should be cleaned prior to use. This material will remove congealed preservatives from new blades and hard-to-remove foreign matter from used blades. Repeat the cleaning procedure by sliding the upper blade in the opposite direction. Wipe blades completely dry. If blades should separate, lift the spring with the fingertip just enough to slide the upper blade into position. Run the motor when reattaching the blade assembly. Apply a thin film of Oster oil and wipe excess oil from blade surfaces prior to use.

Oiling Blades — Apply oil several times during use to maintain a light film on the mating faces of the blades and the tension spring guide. Always wipe excess oil from blade surfaces prior to use.

Sharpening Blades — Dull blades, improper sharpening, and excessive tensioning (to force dull blades to cut) reduce cutting efficiency and can cause the clipper to heat, slow down, and reduce the motor and blade life. When cutting blades no longer cut smoothly and cleanly, sharpening is necessary.

Tape Stripping

Tape stripping (or denuding of the skin) is performed using a tacky transparent tape product such as 1 in. wide Dermiclear (Johnson & Johnson Products, Inc.). After shaving, an assistant holds the animal while successive 10 in. lengths of tape are employed. Successive portions of the length of tape are pressed firmly over the region of skin from which it is desired to remove the outer layer of the epidermis. This is repeated until the skin is observed to become glossy in appearance.

The permeability of guinea pig skin is generally closer to that of the human than either the rabbit or rat (Tregear, 2012).

Anesthesia

Guinea pigs are among the most difficult rodents in which to achieve safe and effective anesthesia, and they are generally considered poor risks for it. Their response to many injectable anesthetics is very variable, and postanesthetic complications such as respiratory infections, digestive disturbances, and generalized depression and inappetence are frequently seen. Many of these problems can be avoided by careful selection of anesthetic agents and a high standard of intra- and postoperative nursing care. At times, guinea pigs may exhibit a peculiar squirming movement during administration of volatile anesthetics. This movement does not signify return to consciousness, and caution should be exercised in administering additional anesthetic (Flecknell, 1987).

Preanesthetic Medication

Guinea pigs are nonaggressive animals that are generally easy to handle and restrain. When frightened they run around their cage at high speed, making safe handling difficult. It is important to approach guinea pigs quietly and handle them gently but firmly. They should be picked up around the shoulders and thorax and the hindquarters supported as they are lifted clear of their cage. Intramuscular or intraperitoneal injection of anesthetic agents can then be carried out. Preanesthetic medication is therefore not usually required, but if an anesthetic is to be administered by intravenous injection into an ear vein, initial sedation is advantageous.

The following drugs can be used to produce sedation and restraint:

1. Fentanyl/fluanisone (1 mL/kg im) will produce restraint, sedation, and sufficient analgesia for minor procedures such as skin biopsy.
2. Diazepam (5 mg/kg ip) produces heavy sedation and immobility, but no analgesia. The animal is easily roused by painful stimuli or other disturbances such as noise. This agent can be useful in providing sufficient sedation to allow local anesthetic techniques to be used humanely.
3. Ketamine (100 mg/kg im) immobilizes guinea pigs but does not produce good analgesia.
4. Alphaxalone/alphadolone (40 mg/kg im) produces deep sedation but requires a large volume of drug to be injected (2–3 mL for an adult guinea pig).
5. Atropine (0.05 mg/kg sc) should be administered prior to any general anesthetic to minimize the volume of bronchial and salivary secretions. It is particularly useful in guinea pigs because of their relatively narrow airways, which are prone to obstruction.

General Anesthesia

Intravenous administration of anesthetics is difficult to achieve in guinea pigs, and drugs are usually administered by the intraperitoneal, subcutaneous, or intramuscular routes. The animals should be carefully weighed and dose rates calculated accurately.

The anesthetic combination of choice is fentanyl/fluanisone together with diazepam or midazolam. This combination provides surgical anesthesia with good muscle relaxation lasting about 45 min. If a longer period of anesthesia is required, further doses can be given (approximately 0.5 mL/kg im every 20–30 min). Following the completion of surgery, the anesthesia can be reversed using naloxone (0.1 mg/kg iv) or buprenorphine (0.1 mg/kg iv).

An effective alternative is to administer ketamine (40 mg/kg ip) and xylazine (5 mg/kg ip). This combination provides about 30 min of surgical anesthesia, although the level of analgesia may be insufficient for major surgery in some animals.

Alphaxalone/alphadolone produces only light surgical anesthesia even when administered by the iv route. If additional anesthetic is administered, severe respiratory depression frequently ensues.

If sodium pentobarbital is to be used, this is best administered at a dose of 25 mg/kg ip to sedate and immobilize the animal, and anesthesia should then be deepened using a volatile agent such as methoxyflurane. Use of the higher dose rates of pentobarbitone (37 mg/kg ip) that are needed to produce surgical anesthesia is frequently associated with an unacceptably high mortality.

Induction of anesthesia by an inhalational agent can be either by use of an anesthetic chamber or administration via a small face mask. Following induction, it is usually most convenient to maintain anesthesia using a face mask, since endotracheal intubation is an extremely difficult technique to carry out in guinea pigs.

Methoxyflurane is the volatile anesthetic of choice in guinea pigs as it has a wide margin of safety and is nonirritant. Halothane can be used successfully, but it can produce profound hypotension even at normal maintenance concentrations. Ether is unsuitable for use in guinea pigs, since it is highly irritant to their respiratory tract, producing increased bronchial

secretions that tend to occlude the narrow airways. In addition, bronchospasm may be produced during induction of anesthesia with ether.

Anesthetic Management

Care must be taken to prevent the development of hypothermia. Postoperative recovery is aided by administering 10–15 mL of warmed dextrose–saline (0%–18% saline, 4% dextrose) sc to correct any fluid deficiency. A warm (25°C–30°C) recovery area should be provided, and the animal given additional subcutaneous fluid for the next few days if its appetite is depressed.

Breeding

Generally, guinea pigs should be bred in the laboratory only if such breeding represents an integral part of the study. This usually means only for reproduction and developmental toxicology studies, for which the guinea pig is an infrequent subject.

Any breeding stock should be obtained from reliable sources. Soon after weaning, any offspring for follow-on generations should be quarantined and placed in the breeding quarters early in order to avoid any breeding setback due to environmental changes.

Puberty in the females may occur in 4–5 weeks; they weigh about 250–400 g at puberty. The males mature at about 8–10 weeks, weighing about 400–600 g. The first mating, however, should be done around 12 weeks of age, when the female and male weigh about 450 and 500 g, respectively. The guinea pig experiences postpartum estrus, and mating at this time will considerably reduce the interval between litters. The gestation period varies between 59 and 72 days, with an average of 63 days. The weight of the guinea pig at birth depends on the nutritional status of the sow and the number of pups in the litter. For single births, the weight may be 150 g; however, for three to four youngsters, the weight is generally between 85 and 90 g. If the live birth weight is below average, the chances of survival are generally very poor. There are usually three to four litters per gestation. Development of young guinea pigs is rapid; they gain 3–5 g of weight per day for the first 2 months. Mature adults may weigh 700–750 g at 5 months of age.

Nonintensive Method

In this procedure, the individual sows (5–10 per boar) may be isolated throughout their breeding span (2 years). The method is wasteful of space, and generally, the annual number of offspring is lower (12 per sow per year). However, it has the advantage of allowing the keeping of correct breeding records, and it is excellent for inbreeding and disease control.

Communal Farrowing

In this system of breeding, the heavily pregnant sows are removed from mating pens and allowed to litter and to rear the young communally. When the youngsters are 180 g in weight, the mothers are removed and returned to mating pens. The preferred system is to have 1 boar and up to 20 sows; the boar can be rotated every week if desired. Such a system yields an average of seven litters per 2-year life span.

Intensive System

Monogamous or polygamous systems can be followed. Monogamous systems are expensive, since a large number of boars have to be maintained, and the yields are lower. In a polygamous system, the yields may be as high as 14–16 young per sow per year.

Developmental Toxicity

Guinea pigs have characteristics that make them unlike any of the other species commonly used for developmental toxicity studies (rabbits, rats, and mice). Their endocrine control of reproduction is similar to that of the human even to its trimester characteristics, and yet pregnancy is preceded by estrus. The placenta is capable of the full range of endocrine activity, but it is labyrinthine and its functions are supplemented by a yolk sac exposed to uterine secretions. Estrus, ovulation, and fertilization can be accurately timed, as with most rodents, and data are available defining or describing most of the aspects of gestation and embryonic development. For example, normal resorption activity involves the loss of approximately 5.8%–6.3% of implanted embryos as determined by differences between the number of implantation sites and functioning corpora lutea (Hoar and King, 1967; Hoar, 1969). Normal embryonic development of the guinea pig through the 26th day of gestation has been described by Scott (1937). The process of ossification of the entire skeleton of the guinea pig has been detailed by Petri (1935). Structural malformations have been induced in guinea pigs in a variety of ways and some occur spontaneously (Edwards, 1957). True preeclampsia has been observed in the guinea pig and thus can be studied in the species (Seidel et al., 1979). Thalidomide given ip as a saline suspension, by gavage, or in dry feed for three consecutive generations produced a "conspicuous number of cleft palates and deformities of the outer ear and shortened limbs" as well as reducing the litter size, increasing prenatal deaths, and producing smaller individual offspring, particularly from those mothers fed thalidomide in the diet (Arbab-Zadeh, 1965).

Trypan blue (an azo dye) produces deleterious effects in the fetus. Pregnant guinea pigs received a single subcutaneous injection of 2 mL of 1% trypan on a single day during the period of days 6–13 of gestation, and their offspring were recovered on day 30 of gestation or allowed to deliver. The response seen at 30 days included an increased resorption rate, growth retardation, and gross abnormalities with the maximum incidence of abnormalities (57%) resulting from injection on day 11. Every embryo from treated females was affected by the dye, with the response varying from shorter crown-rump length to gross abnormalities. The malformations found included cyst of the anterior thoracic wall (49.3%), spina bifida (33.8%), microphthalmia (5.6%), hydrocephaly (4.2%), edema (2.8%), meningocele (1.4%), and other assorted defects (2.8%). Fifty percent of the retarded and/or malformed embryos displayed a posterior cleft palate. Treated females going to term had a reduced litter size, their offspring displaying only those abnormalities (5.3%) that were compatible with life (Hoar and Salem, 1961).

Hypervitaminosis A produces malformations in the guinea pig. Giroud and Martinet (1959a,b) gave pregnant guinea pigs 50,000 IU of vitamin A on days 12–14 of gestation and reported an increased number of spontaneous abortions, resorptions, and a case of mandibular fissure combined with a bifid tongue. Robens (1970) gave guinea pigs 200,000 USP units/kg of vitamin A palmitate as a single dose on selected days (14–20) during organogenesis. Most of the females were allowed to deliver, although some were terminated at 50 days of gestation. Multiple structural defects, involving primarily the head region, were seen in 60.8% of offspring born following maternal treatment on days 14–16. Missing coccygeal vertebrae and agnathia (38.5% of offspring) were seen following treatment on day 17 of gestation. Limb defects (37.2%) were the most frequent abnormalities resulting from treatment on days 18–20, whereas only 1 of 226 control offspring was abnormal.

Edwards (1967, 1969a,b) examined the effects of hyperthermia applied early during gestation on reproduction and feral development in guinea pigs. He noted that resorptions appeared to be most common following hyperthermia on about days 11–15, whereas abortions, occurring at a mean of 32.4 4.85 days of gestation, appeared most frequently (83%) following hypothermia on days 11–18. Of 251 offspring recovered at delivery, the following malformations were noted: microencephaly (41%), hypoplastic digits (13%), exomphalos (7%), talipes (4%), hypoplastic incisors (4%), cataract (3%), renal agenesis (2%), and amyoplasia (2%). A detailed evaluation of prenatal retardation of brain growth at various times during gestation was also conducted by Edwards (1969c). The incidence of

reduced brain weight and microcephaly increased most markedly following hyperthermia for 4 or 8 days during days 15–32 of gestation. Following two successive days of hyperthermia, the effects were most marked on days 20–23 of gestation.

Common Protocols (Immunotoxicology)

As presented in Table 5.11, the guinea pig has been used in a wide variety of studies in toxicology. The most common are the various sensitization, photosensitization studies (Gibson et al., 1983; Parker and Turk, 1983; Thorne et al., 1987; but dating back to Sulzberger, 1930; Rackemann and Simon, 1934; Simon et al., 1934; Simon, 1936) and more recently immunotoxicology assessments (due to their extreme sensitivity; Wilhelmsen and Waag, 2000), through mice are more commonly used due to animal welfare sensitivities. The rationale for the use of guinea pigs in these designs has previously been reviewed (Campbell and Bruce, 1981; Gad and Chengelis, 1989). It should be kept in mind that while these sensitization tests are not required to be terminal, there are substances which can down regulate responsiveness (Andersen, 1985). Typical or prototype protocols for these more common designs are as follows. Other sensitization test designs exist (Chase, 1941, 1953; Landsteiner and DiSomma, 1938; Guillot and Gonnett, 1985; Guillot et al. 1985), as well as a well developed set of respiratory sensitization models (Karol, 1980) and of vaginal sensitization (Maurer et al., 1975, 1980; Newmann et al., 1983).

Modified Buehler Procedure

The modified Buehler procedure is a closed patch procedure for evaluating test substances for potential delayed contact dermal sensitization in guinea pigs. The procedure, based on that

Table 5.11 Dosage Tables for Pharmaceuticals Commonly Used in Guinea Pigs

Antibiotics	
Penicillin—do not administer.	
Cephaloridine—25 mg/kg IM daily.	
Sulfamethazine—333 mg/L drinking water for 300 g animal.	
Tetracycline—255 mg/L drinking water for 300 g animal.	
Antihistamines	
Diphenhydramine (Benadryl)	5.0 mg/kg sc
Tripelennamine	5.0 mg/kg po or im
Tranquilizers	
Chlorpromazine	0.5 mg/kg im
Promazine HCl	0.5–1.0 mg/kg im
Meprobamate	100 mg/kg im
Anesthetics	
Pentobarbital sodium	30 mg/kg iv; 40 mg/kg ip
Thiopental sodium	20 mg/kg iv; 55 mg/kg ip
Ketamine	22–44 mg/kg im
Droperidol-fentanyl	0.66–0.88 mg/kg im
Urethane	1500 mg/kg ip
Analgesics	
Aspirin	269 mg/kg ip
Meperidine	2 mg/kg im
Miscellaneous	
Atropine	0.05 mg/kg sc, im
Heparin	5 mg/kg iv

described by Buehler (1965), is practical for test substances that cannot be evaluated by the traditional intradermal injection procedure of Landsteiner and Jacobs or by the guinea pig maximization test (GPMT) for skin sensitization testing. The closed patch procedure is performed when a test substance either is highly irritating to the skin by the intradermal injection route of exposure or cannot be dissolved or suspended in a form allowing injection. It is also the method of choice for some companies. This procedure, which is one version of the Buehler test, complies with the test standards set forth in the Toxic Substances Control Act (TSCA) and other regulatory test rules. There are other versions that also comply. While generally not favored for the evaluation of drug or medical devices, Buehler-style tests still have utility for screening and identify moderate to strong sensitization.

Animals

1. Young albino female Hartley strain guinea pigs, weighing between 300 and 400 g, are currently the standard animals used. Studies sponsored by the Charles River Laboratories have shown that their hairless guinea pigs are just as responsive, however.
2. Although several proposed test rules suggest the use of male guinea pigs, the female sex is preferred because the aggressive social behavior of males may result in considerable skin damage that might interfere with the interpretation of challenge reactions. This concern occurs because animals are commonly group housed (Marzulli and Maibach, 1983).
3. Animals that show poor growth or are ill in any way are not used, since illness can markedly decrease the response. Animals with skin marked or scarred from fighting are avoided. The guinea pigs are observed for at least 2 weeks prior to test to detect any illness before starting a study.
4. The guinea pigs are identified by a cage card and marking pen or any other suitable method. There is no regulatory requirement, however, for the identification of individual animals.
5. The guinea pigs are randomly assigned to test and negative control groups consisting of at least 15 and 6 animals each, respectively. If a pretest group is necessary, as many animals as needed for that group are randomized also.

Pretest Screen

1. If practical, the dermal irritation threshold concentration should be established for the test substance prior to the first induction application. A concentration of the test substance that produces minimal or no irritation (erythema and/or edema formation) is determined. The highest concentration that produces no irritation is preferred for the dermal sensitization study challenge dose.
2. Those animals randomly assigned to the pretest group are used.
3. Each animal is prepared by clipping a 1 in.^{2} area of hair from the left upper flank using a small animal clipper with a size 40 blade.
4. The test substance is diluted, emulsified, or suspended in a suitable vehicle. Vehicles are selected on the basis of their solubilizing capacity for the test substance and on their ability to penetrate the skin.
5. Different concentrations of the test substance are tested on the pretest group of guinea pigs; a few animals are used for each concentration tested.
6. A volume of 0.5 mL is applied to a patch consisting of a cotton pad (1 in. × 1 in.) occluded with impermeable surgical tape, or placed in a Hilltop-style occlusive "chamber."
7. The patch is applied to the shaved left flank of a guinea pig. The patch is held firmly in place for 24 hours by wrapping the trunk of the animal with a 3 in. wide elastic bandage. A 2 in. wide strip of tape is used to line the center adhesive side of the bandage in order to prevent skin damage from the adhesive.
8. After 24 hours of exposure, the wrappings and patches are removed.
9. Observations of skin reactions (erythema and/or edema formation) are recorded 48 hours after application.
10. A judgment is made as to which concentration will be used for the dermal sensitization study based on the dermal irritation data that have been collected. The highest concentration that produces minimal or no dermal irritation is selected.

Induction Phase

1. Test group and control group guinea pigs are weighed at the beginning of the study and weekly thereafter.
2. Test control group guinea pigs are shaved as described earlier.
3. If the test substance is a liquid (solution, suspension, or emulsion), a volume of 0.5 mL of the highest concentration found to be nonirritating in a suitable vehicle (as determined in the pretest portion of this procedure) is applied to a patch consisting of a cotton pad (1 in. × 1 in.) occluded with impermeable surgical tape. If the test substance is a solid or semisolid, 0.5 g* is applied. If the test substance is a fabric, a 1 in.^{2} is moistened with 0.5 mL of physiological saline before application.
4. The first induction patch is applied to the clipped left flank of each test group guinea pig. The patch is held firmly in place for 24 hours by wrapping the trunk of each animal with a 3 in. wide elastic bandage. A 2 in. wide strip of tape is used to line the center adhesive side of the bandage in order to prevent skin damage from the adhesive. A 2 in. length of athletic adhesive tape is placed over the bandage wrap as a precautionary measure to prevent unraveling.
5. After 24 hours of exposure, the wrappings and patches are removed and disposed of in a plastic bag.
6. Each dermal reaction, if any, is scored on the basis of previously designated values for erythema and edema formation such as the Draize scale (Draize et al., 1959; see Table 5.12). Observations are made 48 hours after initiation of the first induction application. Resulting dermal irritation scores are recorded.
7. After the initial induction application, subsequent induction applications (2–9) are made on alternate days (3 times weekly) until a total of 10 treatments has been administered. Each of these patches is removed after 6 hours of exposure. It should be noted that some investigators use a modification that calls for one application per week for 3 weeks.
8. Observations are made 24 and 48 hours after initiation of each subsequent induction application. Dermal scores of the remaining nine induction applications are recorded.
9. Clipping the hair from the left flank induction sites of test group animals and corresponding sites on negative control group animals is performed just prior to each subsequent induction application. Only the test group guinea pigs receive the induction applications.

Table 5.12   Evaluation of Skin Reactions

Skin Reaction	Value
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar	4
Formation (injuries in-depth)	
Necrosis (death of tissue)	+N
Eschar (sloughing)	+E
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema 9 raised more than 1 mm and extending beyond the area of exposure)	4

Source: Draize, J. H., *The Appraisal of Chemicals in Foods, Drugs and Cosmetics*, Association of Food and Drug Officials of the U.S., Austin, TX, 1959, pp. 36–45.

* When the test substance is in flake, granule, powder, or other particulate form, the weight of the test substance that has a volume of 0.5 mL (after compacting as much as possible without crushing or altering the individual particles, such as by tapping the measuring container) is used whenever this volume weighs less than 0.5 g.

Challenge Phase

1. Fourteen days after the tenth induction application, all 10 test group and 3 of 6 control group guinea pigs are prepared for challenge application by clipping a 1 in.^{2} of hair from the right side (the side opposite that which was clipped during the induction phase).
2. A challenge dose, using freshly prepared test substance (solution, suspension, emulsion, semisolid, solid, or fabric), is applied topically to the right side (which had remained untreated during the induction application) of test group animals. The left side, which had previously received induction applications, is not challenge dosed.
3. The concentration of the challenge dose is the same as that used for the first induction application. (It must be a concentration that does not produce dermal irritation after one 24-hour application.)
4. Each of three negative control group guinea pigs is challenge dosed on the right flank at approximately the same time that the test group guinea pigs are challenge dosed. This is, in effect, a check for unexpected primary irritation.
5. All patches are held in contact with the skin for 24 hours before removal.
6. The skin sites are evaluated using the previously selected scoring system for erythema and edema formation, such as that presented in Table 5.12. Observations are made 48, 72, and 96 hours after initiation of the challenge application and the skin reactions are recorded.

Rechallenge Phase

1. If the test substance is judged a nonsensitizing agent after the first challenge application, or causes dermal sensitization in only a few animals, or causes dermal reactions that are weak or questionable, then a second and final challenge application will be performed on each test animal 7 days after the initiation of the first challenge dose.
2. Controls from the first challenge application are not rechallenged because they have been exposed to the test substance and are no longer true negative controls. The three remaining naive control group animals (not used for the first challenge) are challenged for comparison to the test group animals.
3. The procedure used for the first challenge applications will be used for the second challenge application (including reclipping, patching method, and duration of exposure). Either the same concentration or a new concentration (higher or lower) of test substance may be used, depending on the results of the first challenge. Observations are made 48, 72, and 96 hours after initiation of the rechallenge application and skin reactions are recorded.
4. When a rechallenge application is performed, the data from both challenges are compared. If neither challenge produces a positive dermal reaction, the classification of the test substance is based on both challenge applications. If one challenge application (whether it is the first or second) produces a greater number of positive dermal reactions than the other, the classification of the test substance is based on the challenge with the most positive responses.
5. Two or more unequivocally positive responses in a group of IS animals should be considered significant. A negative, equivocal, or single response probably assures that a substance is not a strong sensitizer, although this is best confirmed by further testing with human subjects (NAS, 1977).

Interpretation of Results

1. Judgment concerning the presence of absence of sensitization is made for each animal. The judgment is made by comparing the test animal's challenge responses to its first induction treatment response as well as to those challenge responses of negative control animals.
2. Challenge reactions to the test substance that are stronger than challenge reactions to negative controls, or to those seen after the initial induction application should be suspected as results of sensitization (NAS, 1977). A reaction that occurs at 48 hours, but resolves by 72 or 96 hours, should be considered a positive response as long as it is stronger than that which is displayed by controls at the same time interval.

Strengths and Weaknesses

There are a number of both advantages and disadvantages to the Buehler methodology, which has been in use for over 20 years (Griffith and Buehler, 1977; Griffith et al., 1981, 1983). The relative importance and merits of each depend on the intended use of the material. The four advantages are as follows:

1. Virtually no false positives (in fact, in the experience of the author, when the pretest is properly conducted, there are no false positives), compared to human experience, are generated by test.
2. The techniques involved are easy to learn and very reproducible.
3. The Buehler-style test does not overpredict the potency of sensitizers. That is, materials that are identified as sensitizers are truly classified as very strong, weak, or in between—not all (or nearly all) as very strong.
4. There is a large database in existence for the Buehler-style test. Unfortunately, the vast majority is not in the published literature.

Likewise, there are three disadvantages associated with the Buehler-style test:

1. The test gives a high rate of false negatives for weak sensitizers and a detectable rate of false negatives for moderate sensitizers. That is, the method is somewhat insensitive—particularly if techniques for occlusive wrapping are inadequate.
2. The test takes a long time to complete. If animals are on hand when started, the test is 5–6 weeks long. As few laboratories keep a "pool" of guinea pigs on hand (especially as they are the most expensive of the common lab species), the usual case is that 8–10 weeks is the minimum time required to get an answer from this test.
3. The test uses a relatively large amount of test material. In the normal acute "battery," the guinea pig test systems use more material than any other test systems unless an acute inhalation study is included. With 10 induction applications, this is particularly true for the Buehler-style test.

Guinea Pig Maximization Test

The GPMT was developed by Magnusson and Kligman (1969, 1970) and Magnusson (1975) and is considered a highly sensitive procedure for evaluating test substances for potential dermal sensitization.

While an alternative test using mice (the local lymph node assay [LLNA]) was developed and widely incorporated into regulatory schemes, its limitations (Kreilinga et al., 2008) have led to retention and preference for the GPMT in several regulatory guidances.

Animals

1. Young adult female guinea pigs weighing between 250 and 350 g at the initiation of the study are used.
2. Animals that show poor growth or are ill in any way are not used, since illness may markedly decrease the response. Animals with skin marked or scarred from fighting are avoided. The guinea pigs are observed for at least 2 weeks to detect any illness before starting a study.
3. The guinea pigs are randomly assigned to two groups: (1) a test group consisting of 15 animals and (2) a control group consisting of 6 animals. If a pretest group is necessary, as many animals as needed for that group also are randomized.
4. Test and control group guinea pigs are weighed 1 week prior to dosing (day 7), on the day of dosing (day 0), and weekly thereafter.

Pretest

1. Several animals are used to pretest the test substance and vehicles to determine the topical dermal irritation threshold concentration.
2. These animals are shaved on the left flank, to which is applied a 2 cm × 2 cm filter paper patch that contains 0.1 mL of the test concentration.

3. The trunks of the animals are wrapped for 24 hours with a 3 in. wide elastic bandage to hold the patch in contact with the skin.
4. Wrappings are removed after the 24 hours exposure, and based on skin reactions at 48 hours, a concentration of the test substance to be used on the test is determined. Dermal irritation values are recorded for future reference.
5. In addition, several guinea pigs are utilized to determine a concentration (generally between 1% and 5%) of test substance in vehicle and in Freund's Complete Adjuvant (FCA) emulsion that can be injected intradermally without eliciting a strong local or systemic toxic reaction.
6. The hair is clipped in an area of approximately 4 cm × 6 cm from the upper shoulder region of these animals.
7. Several concentrations of the test substance (ranging between 1% and 5%) can be injected in the same animal to compare local dermal reactions produced by the different concentrations.
8. However, if systemic toxicity is suspected, then each concentration should be tested in separate animals to determine local and systemic effects.
9. The dermal reactions (erythema, edema, and diameter) are recorded 24 hours after the id injections.

Induction Stage 1 (Day 0)

1. The hair in an area of 4 cm × 6 cm is clipped from the shoulder region of each test and control group guinea pig on day 0.
2. Three pairs of intradermal (id) injections are made with a glass 1 mL tuberculin syringe with a 26-gauge needle, each pair flanking the dorsal midline.
3. The three pairs of id injections for test group animals are as follows:
   a. 0.1 mL test substance in appropriate vehicle
   b. 0.1 mL FCA emulsion alone
   c. 0.1 mL test substance in FCA emulsion
4. The three pairs of id injections for control group animals are as follows:
   a. 0.1 mL vehicle alone
   b. 0.1 FCA emulsion alone
   c. 0.1 mL vehicle in FCA emulsion
5. Injections (a) and (b) in the two aforementioned steps are given close to each other and nearest the head; injection (c) is given most posteriorly.
6. The date, time, and initials of those individuals performing the id injections are recorded.
7. Immediately before injection, an emulsion is prepared by blending commercial FCA with an equal volume of house distilled water or other solvent as appropriate.
   a. Water-soluble test materials are dissolved in the water phase prior to emulsification.
   b. Oil-soluble or water-insoluble materials are dissolved or suspended in FCA prior to adding water.
   c. Paraffin oil, peanut oil, or propylene glycol can be used for dissolving or suspending water-insoluble materials.
   d. A homogenizer is used to emulsify the FCA alone and the test substance in either FCA or vehicle prior to the id injections.
   e. The concentration of the test substance for id injections is adjusted to the highest level that can be well tolerated locally and generally.
8. The adjuvant injection infiltration sometimes causes ulceration, especially when the injection is superficial. This ulceration lasts several weeks. These lesions are undesirable but do not invalidate the test results except for lowering the threshold level for skin irritation.

Induction Stage 2 (Day 7)

1. Test substance preparation:
   a. The concentration of the test substance is adjusted to the highest level that can be well tolerated.
   b. If the test substance is an irritant, a concentration is chosen that causes a weak to moderate inflammation (as determined by the pretest).

    c.   Solids are micronized or reduced to a fine powder and then suspended in a vehicle, such as petrolatum or propylene glycol.
    d.   Water- and oil-soluble test substances are dissolved in an appropriate vehicle.
    e.   Liquid materials are applied as such, or diluted if necessary.

2. The same area over the shoulder region that received id injections on day 0 is again shaved on both test and control guinea pigs.
3. A volume of 0.3 mL of a mildly irritating concentration (if possible) of the test substance (determined by the pretest) is spread over a 1 in. × 2 in. filter paper patch in a thick, even layer.
4. The patch is occluded with surgical tape and then is secured to test group animals with an elastic bandage, which is wrapped around the torso of each test group animal.
5. The control group animals are exposed to 0.3 mL of 100% vehicle using the same procedure.
6. The date, time, and initials of those individuals performing the second induction should be recorded.
7. The dressings of both groups are left in place for 48 hours before removal.

Challenge Stage (Day 21)

1. An area of hair (1.5 in. × 1.5 in.) on both flanks of the guinea pigs (15 test and 3 controls) is shaved.
2. A 1 in. × 1 in. patch with a nonirritating concentration of test substance in vehicle (as determined by the pretest) is applied to the left flank and a 1 in. × 1 in. patch with 100% vehicle is applied to the right flank.
3. The torso of each guinea pig is wrapped in an elastic bandage to secure the patches for 24 hours.
4. The date, time, and initials of those individuals performing the challenge dose are recorded.
5. The patches are removed 24 hours after application.

Rechallenge (Day 28)

1. If the first challenge application of test substance does not cause dermal sensitization, or causes dermal sensitization in only a few animals, or causes dermal reactions that are weak or questionable, then a second challenge application of test substance to the 15 test group guinea pigs should be conducted on day 28 (week after the first challenge). The three remaining naive control group animals (not used for the first challenge) are challenged for comparison to the test group animals.
2. The three negative control group animals used on day 21 will not be rechallenged. These animals will be discontinued from the study because they were exposed to the test substance during the first challenge and are no longer negative controls.
3. A 1 in. × 1 in. patch with a nonirritating concentration of test substance in vehicle is applied to the right flank of test and control group animals. The left flanks are not dosed.
4. The date, time, and initials of those individuals performing the rechallenge dose should be recorded.
5. Steps 3 and 5 are followed as for challenge stage (day 21).

Observations: Challenge and/or Rechallenge Readings

1. Twenty-one hours after removing the patch, the challenge area on each flank is cleaned and shaved, if necessary.
2. Twenty-four hours after removing the patch, the first reading of dermal reactions is taken.
3. The dermal reactions are scored on a four-point scale:
    a.   0—No reaction
    b.   1—Scattered and mild redness
    c.   2—Moderate and diffuse redness
    d.   3—Intense redness and swelling
4. Forty-eight hours after removing the patch, the second reading is taken and the scores are recorded.

Interpretation of Results

1. Both the intensity and duration of the test responses to the test substance and the vehicle are evaluated.
2. The important statistic in the GPMT is the frequency of sensitization and not the intensity of challenge responses. A value of 1 is considered just as positive as a value of 3 (as long as the values for controls are zero).
3. The test agent is a sensitizer if the challenge reactions in the test group clearly outweigh those in the control group. A reaction that occurs at 24 hours but resolves by 48 hours after removal of patches should be considered a positive response, as long as it is stronger than that which is displayed by controls. The sensitization rate (% of positive responders) is based on the greatest number of animals showing a positive response, whether it is from the 24 hours data or the 48 hours data after removal of patches.
4. When a second challenge application is performed, the data from both challenges are compared. If neither challenge produces a positive dermal reaction, the classification of the test substance is based on both challenge applications. If one challenge application (whether it is the first or second) produced a greater number of positive dermal reactions than the other, the classification of the test substance is based on the challenge with the most positive responses.
5. Under the classification scheme of Kligman (1966) (see Table 5.13), the test substance is assigned to one of five classes, according to the percentage of animals sensitized, ranging from a weak Grade I to an extreme Grade V.

The advantages and disadvantages of the GPMT can be summarized as follows. First, the advantages:

1. The test system is sensitive and effectively detects weak sensitizers. It has a low false-negative rate.
2. If properly conducted, there are no false positives—that is, materials that are identified as potential sensitizers will act as such at some incidence level in humans.
3. There is a large database available on the evaluation of compounds in this test system, and many people are familiar with the test system.

The disadvantages, meanwhile, are as follows:

1. The test system is sensitive; it overpredicts potency for many sensitizers. There is no real differentiation between weak, moderate, and strong sensitizers; virtually all positive test results identify a material as strong.
2. The techniques involved (particularly the intradermal injections) are not easy. Some regulatory officials have estimated that as many as 35% of the laboratories that try cannot master the system to get it to work reproducibly.
3. The test, though not as long as the Buehler-style test, still takes a minimum of 4 weeks to produce an answer.
4. The test uses a significant amount of test material.

Table 5.13   Sensitization Severity Grading Based on Incidence of Positive Responses

Sensitization Rate (%)	Grade	Classification
0–8	I	Weak
9–28	II	Mild
29–64	III	Moderate
65–80	IV	Strong
81–100	V	Extreme

Source: Kligman, A. M., *J. Invest. Dermatol.*, 47, 393, 1966.

5. One cannot evaluate fibers or other materials that cannot be injected (such as either solids that cannot be finely ground and/or suspended or that are highly irritating or toxic by the iv route).
6. The irritation pretest is critical. Failure to detect irritation in this small group of animals does not guarantee against irritation in test animals at challenge.

Split Adjuvant Test

The guinea pig split adjuvant dermal sensitization procedure for detecting contact allergenicity is based on that developed by Maguire and Chase (1967, 1972) and Maguire (1973a,b, 1975) and is sensitive and effective for the detection of substance and products with weak allergic potential and will serve as a useful alternative for testing materials that cannot be injected intradermally (e.g., fabrics, nonsoluble solids, and extremely irritating or toxic materials). A concise outline of the split adjuvant technique has been published (Klecak, 1983).

Animals

1. Young adult female guinea pigs weighing between 250 and 350 g at the initiation of the study are used.
2. Animals that show poor growth or are ill in any way are not used, since illness markedly decreases the response. Animals with skin marked or scarred from fighting are avoided. The guinea pigs are observed for at least 2 weeks to detect any illness before starting a study.
3. The guinea pigs are randomly assigned to two groups: (1) a test group consisting of 10 animals and (2) a control group consisting of 6 animals. If a pretest group is necessary, as many animals as needed for that group also are randomized.
4. Test and control group guinea pigs are weighed and the weights are recorded 1 week prior to dosing (day 7), on the day of dosing (day 0), and weekly thereafter.

Pretest

1. Several animals are used to pretest the test substance and vehicles to determine the dermal irritation threshold concentration.
2. These animals are shaved on the left flank (2 cm × 2 cm).
3. Then 0.2 mL of ointment (semisolid) or 0.1 mL of liquid is spread onto a 1.5 cm × 1.5 cm Whatman No. 3 filter paper patch, which is occluded on the opposite side with surgical tape.
   a. Solid test substances are micronized or reduced to a fine powder and then suspended in a vehicle, such as petrolatum or propylene glycol.
   b. Water- and oil-soluble test substances are dissolved in an appropriate vehicle.
   c. Liquid test substances are applied as such (100%) or diluted if necessary.
4. The pretest patch is then applied to the left flank. The trunk of each animal is wrapped for 24 hours with a 3 in. wide elastic bandage to hold the patch in contact with the skin.
5. Wrappings are removed after 24 hours of exposure. Based on skin reactions at 48 hours, a concentration of the test substance to be used on test is determined. Dermal irritation values are recorded for later reference.

Induction Stage

The date, time, and initials of those individuals performing the induction applications should be recorded.

Day 0

1. The hair in an area 1 in. × 1 in. is clipped behind the shoulder girdle of each test and control group guinea pig.

2. Dry ice is applied for 5 s to the skin site of each test and control animal. Dry ice is used only for the day 0 induction application.
3. Then 0.2 mL of ointment (semisolid) or 0.1 mL of liquid is spread onto a 0.5 in. × 0.5 in. Whatman No. 3 filter paper patch, which is occluded on the opposite side with surgical tape.
    a. The test substance is tested at a concentration that is minimally irritating (if possible), as determined by the pretest.
    b. If the substance is mixed in petrolatum or ointment, 0.2 mL is dispensed onto the patch.
    c. In the case of liquids, 0.1 mL is used.
    d. If a fabric is to be tested, a 1.5 cm × 1.5 cm sample is cut, moistened with 0.2 mL of physiological saline, and then is applied under a filter paper patch.
4. Control group animals are dosed with vehicle only, not test substance.
5. The trunk of each animal is wrapped for 48 hours with a 3 in. wide elastic bandage to hold the patch in contact with the skin.

Day 2

1. The wrapping and patch are removed from each test and control group animal 48 hours after the initial induction application.
2. A fresh patch is applied to the same site using the same procedure as described for induction day 0 (without dry ice). Test group animals receive test substance in vehicle, and control group animals receive vehicle alone for a 48-hour period.

Day 4

1. The wrapping and patch are removed from each test and control group animal 48 hours after the application of the day 2 induction patch.
2. An emulsion of FCA emulsion is prepared by blending commercial FCA with an equal volume of house distilled water.
3. Two volumes of 0.1 mL of FCA emulsion are injected id into the induction site of each test and each control group animal with a glass 1 mL tuberculin syringe and a 26-gauge needle. These two injections flank the dorsal midline.
4. A fresh patch is applied to the same site using the same procedure as described on induction day 2. Test group animals receive test substance in vehicle, and control group animals receive vehicle alone for a 48-hour period.

Day 6

The wrapping and patch are removed from each test and control group animal 48 hours after the application of the day 4 induction patch.

Day 7

A fresh patch is applied to the induction site using the same procedure as described on induction day 2. Test group animals receive test substance in vehicle, and control group animals receive vehicle alone for a 48-hour period.

Day 9

The wrapping and patch are removed from each test and control group animal 48 hours after the application of the day 7 induction patch.

Challenge Stage (Day 21)

1. An area of hair (1 in. × 1 in.) on both flanks of the guinea pigs (10 test and 3 controls) is shaved.
2. A 0.5 in. × 0.5 in. filter paper patch with the highest nonirritating concentration of test substance in vehicle (as determined by the pretest) is applied to the left flank, and 1.5 cm × 1.5 cm patch with 100% vehicle is applied to the right flank.
    a. If the test substance is liquid, 0.1 mL is applied to the patch.
    b. If the test substance is mixed in petroleum or ointment, 0.2 mL is dispensed onto the patch.
    c. If the test substance is a fabric, a 0.5 in. × 0.5 in. sample is cut, moistened with physiological saline, and then is applied under a patch.
3. The torso of each guinea pig is wrapped with an elastic bandage to secure the patches for 24 hours.
4. The date, time, and initials of those individuals performing the challenge dose should be recorded.
5. The patches are removed on day 22 and the challenge area on each flank is cleaned and clipped atraumatically.

Challenge Readings

1. On day 23, 24 hours after removing the patch, the first reading of dermal reactions is taken, and results are recorded.
2. Readings of the challenge site are taken again 48 hours after removing the patch, and results are recorded.
3. The intensity of the skin reaction is classified according to the following rating scale used by Maguire (1973b):
    0 = Normal skin
    + = Very faint, nonconfluent pink
    + = Faint pink
    . ++ = Pale pink to pink, slight edema
    +++ = Pink, moderate edema
    ++++ = Pink and thickened
    +++++ = Bright pink, markedly thickened

Interpretation of Results

1. The frequency, intensity, and duration of the test responses to the test substance and the vehicle are evaluated.
2. The test substance is a sensitizer if the challenge reactions in the test group clearly outweigh those in the control group.
3. Two or more unequivocally positive responses (at least a + on the rating scale) in a group of 10 animals should be considered significant. A negative, equivocal, or single response probably assures that a substance is not a strong sensitizer, although this is best confirmed by further testing with human subjects (NAS, 1977).

Rechallenge (Day 28)

1. If the first challenge application does not cause dermal sensitization, then a second application of the 10 test group guinea pigs will be conducted on day 28 (1 week after the first challenge). The remaining naive control group animals (not used for the first challenge) are challenged for comparison to the test group animals.
2. The three negative control group animals used on day 21 will not be rechallenged. These animals will be discontinued from the study because they were exposed to the test article during the first challenge and are no longer negative controls.

3. A 0.5 in. × 0.5 in. patch with the highest nonirritating concentration of test substance in vehicle is applied to the right flank of test and control group animals. The left flanks are not dosed.
4. The date, time, and initials of those individuals performing the rechallenge dose are recorded.
5. Steps 3 and 5 of the challenge procedure are repeated here.

Strengths and Weaknesses

The advantages and disadvantages of the split adjuvant test can be summarized in the following text. The advantages are as follows:

1. The test system has a lower false-negative rate for moderate and weak sensitizers than does the Buehler design.
2. If properly conducted, there are no false positives.
3. Fibers and other materials that cannot be injected intradermally can be evaluated here.

As elsewhere, there is also a list of disadvantages. These include the following:

1. The techniques involved (particularly the intradermal injection) are not easy ones.
2. The sensitivity of the test system is "bought" at the expense of making relative hazard predictions not necessarily accurate. The test system tends to overpredict potency.
3. The test still both takes a relatively long time to complete and uses a significant amount of test material.
4. There is a limited published database on test system performance, and relatively few people have experience with it.

Photosensitization Tests

There are at least five in vivo photosensitization test methods. Only two of these (Harber and Shalita method and the Armstrong assay) will be presented here. The other three (the Vinson and Borselli method, the Guillot et al. method, and a method using mice) are beyond the scope of this text.

Though the pattern of evolution of predictive animal tests is not as clear as that of dermal sensitization tests, the two methods presented here each represents a distinct phase of that development. The first reported use of the guinea pig to study photosensitization was for sulfanilamide (Epstein, 1939).

Harber and Shalita Method

This is the older of the two methods and uses dermal exposure without any adjuvant to increase the response. This method was originally published by Harber and Shalita in 1975.

Animals

1. Young adult female Hartley strain guinea pigs weighing between 300 and 400 g at the start of the study are used.
2. Animals that show poor growth or are ill in any way are not used, since illness markedly decreases the response. Animals with skin marked or scarred from fighting are avoided. The guinea pigs are quarantined and observed for at least 2 weeks to detect any illness before starting a study.
3. The guinea pigs are randomly assigned to a test group of 10 animals and negative control group of 6 animals. If a pretest group is necessary, as many animals as needed for that group also are randomized.
4. Test and control group guinea pigs are weighed 1 week prior to dosing (day 7), on the day of dosing (day 0), and weekly thereafter.

Pretest (If Necessary)

1. Several animals are used to pretest different concentrations of test substance in vehicle (usually acetone) to determine the topical dermal irritation threshold concentration on skin that is exposed to ultraviolet B (UVB) and ultraviolet A (UVA) irradiation sequentially and on skin that is exposed to UVA irradiation alone.
2. The hair of these animals is shaved over the whole dorsal region.
3. A volume of 0.2 mL of each test concentration is applied twice to each guinea pig: (1) to the nuchal region and (2) to the dorsal lumbar region.
4. Thirty minutes after application, the treated nuchal sites are irradiated with sunlamp emissions (UVB) for 30 min, while the lumbar sites are shielded with Elastoplast tape.
5. After the UVB exposure, the tape is removed from the lumbar region, and both the treated nuchal sites and lumbar sites are irradiated with black light emissions (UVA) for 30 min.
6. The animals are returned to their respective cages after the UVA exposure.
7. Twenty-four hours after the initial exposure to the test substance, the nuchal and lumbar skin sites are scored for erythema formation.
8. A concentration is chosen for induction applications that causes a mild or weak erythema response at the nuchal sites. If the test substance does not cause an erythema response, then the highest concentration level that is practical should be used for induction.
9. The highest concentrate of the test substance that is non-irritating to test sites is used for challenge application. Two lower concentrations of the test substance, prepared by serial dilution from the hint concentration, are also used for the challenge application.

Induction Stage (Days D, 2, 4, 7, 9, II)

1. The hair in an area of approximately 1 in. × 1 in. is clipped from the nuchal region of each test and control group guinea pig.
2. A volume of 0.2 mL of a relatively high concentration of the test substance in either acetone or ethanol is applied to the shaved nuchal region of each test group guinea pig. The concentration will be the highest level that can be well tolerated locally and generally by the guinea pig, as determined by a pretest for dermal irritation.
3. A volume of 0.2 mL of solvent (acetone or ethanol) is applied to the shaved nuchal region of each control group guinea pig.
4. Thirty minutes after application, the treated nuchal sites of test and control guinea pigs are irradiated with sunlamp emissions for 30 min and black light emissions for 30 min, successively. The lumbar region of the back is shielded from the light sources during the irradiation procedures with an elastic bandage that is wrapped around the torso of each animal.
5. The clipping, topical exposure to test substance, and irradiation procedures are repeated six times during a 12-day period (typical study days are 0, 2, 4, 7, 9, and 11).

Challenge Stage (Day 32)

1. Elicitation of contact photosensitivity is performed 21 days from the last sensitizing (induction) exposure.
2. The hair of the dorsal lumbar region of each of 10 test group and 3 of 6 control group guinea pigs is clipped for the first time.
3. Three different concentrations of test substance using the solvent used for induction, as determined from the pretest, are applied topically to this region; test and control animals are treated alike. Each concentration is applied to the right and left side of the dorsal midline.
4. The torso of each test and control guinea pig is wrapped in Saran wrap (1 layer thick) after the test chemical is applied. The Saran wrap is held in place at the ends with athletic adhesive tape. The same tape is used to shield the left side of each animal from the UVA light source.

5. Thirty minutes after application, the right side of each animal is exposed to nonerythrogenic (>320 rim) UVA emissions for 30 min. The radiation is passed through a pane of window glass 3 mm thick in order to eliminate passage of radiation lower than 320 nm.
6. After the black light exposure, all animals are unwrapped, returned to their respective cages, and placed in a darkened room for 24 hours.

Challenge Readings

1. If the test substance leaves a colored residue, the excess test material is removed by washing with a suitable solvent at 24 hours so that the area of challenge skin can be evaluated accurately.
2. All test sites, both irradiated and nonirradiated, are scored and interpreted 24 and 48 hours after the initial test substance application and subsequent exposure to black light irradiation.
3. Erythema is scored as follows:
   a. 0—No erythema
   b. 1—Minimal, but definite erythema
   c. 2—Moderate erythema
   d. 3—Considerable erythema
   e. 4—Maximal erythema
4. Erythema scores are recorded.

Rechallenge

1. If the test substance is judged a nonphotosensitizing agent after the first challenge application, a second and final challenge application will be performed on each test group animal 7 days after the initiation of the first challenge dose.
2. Controls from the first challenge application are not rechallenged because they have been exposed to the test substance and are no longer true negative (naive) controls. The three remaining naive control group animals (not used for the first challenge) are challenged for comparison to the rechallenge of test group animals.
3. The procedure used for the first challenge application will be used for the second application, either the same or a new concentration of test substance, including reshaving, the same patching method, and the same duration of exposure. Observations are again made 24 and 48 hours after the second challenge application and skin reactions are recorded.

Interpretation of Results

1. The negative control group of animals, having received no previous photosensitive (induction) exposures, serves to identify any phototoxic or primary irritant (nonphototoxic) substances.
2. An erythema score of 1 or more is considered a positive response.
3. Interpretation of data is based on the dermal score for erythema (see Gad and Chengelis, 1989, for details).

Armstrong Assay

This method, originally published by Ichikawa et al. (1981) and Harber (1981), introduced the use of adjuvants in a photosensitization test system.

This assay has been recommended by the Cosmetic, Toiletries and Fragrances Association. It is of interest that the Environmental Protection Agency (EPA) has not made public a concern about photoallergens, since several pesticides have similar chemical structures to fragrances and numerous pesticides are known to form reactive species in the presence of UV light.

Lights — The Armstrong assay uses UVA light (320–100 nm) in the induction and challenge phase. The UVA lights are commonly known as "black lights" and can be purchased as "BLB" fluorescence-type bulbs from major lighting manufacturers. However, the selection of the light source is critical, since the range of wavelengths emitted by the bulb is controlled by the phosphor coating and different manufacturers use different phosphors to produce BLB lights. There may even be different phosphors used by the same manufacturer, and there is no code on the bulbs to indicate which phosphor is being used. The General Electric BLB emits effective energy only at wavelengths longer than 350 nm, whereas the entire spectrum between 315 and 400 nm is covered by the Sylvania BLB bulb. Less than 2% of the total energy emitted by the General Electric BLB light is between 250 and 350 nm, whereas 42% of the energy from the Sylvania BLB light falls in this range. There are known photoallergens that require the energy contained in the spectrum below 345 nm for activation and thus give a false negative if the incorrect light source is used. The best precaution is to determine the emission spectrum of the light source that is to be used in the assay.

It is necessary to determine the total energy being emitted by the lights in order to calculate the proper joules per square centimeter (J/cm^2) exposure. An International Light Model 700 provides a relatively inexpensive means of measuring the light energy when fitted with a cosine-corrected UVA detector (W150s quartz diffuser, UVA pass filter SEE015 detector). The device has a peak sensitivity of 360 nm and a width of 50 nm. A bank of eight bulbs is readily prepared by bolting together two industrial four-bulb (48 in. long) reflectors. Two sets of these will allow 40 animals to be treated at one time. The lights are allowed to warm 30 min before use. They are turned off just before the animals are placed under them and then turned back on. The light intensity is measured at several locations at the level of the top of the backs of the animals and the correct exposure time then calculated. The lights are adjusted to be between 4 and 6 in. above the back and 10 J/cm^2 is the proper exposure.

Patching — The Hill Top Chamber (see the earlier description of the Buehler assay) provides a good patching system in this assay. A volume of 0.3 mL is used. The animal restrainers described in the description of the Buehler assay work well for holding the animals during the patching and the exposure to the light as well as in providing excellent occlusion.

Induction Site Preparation — The majority of hair is removed from the intended patching site with a small animal clipper fitted with a #40 blade. The assay has a frequent requirement for the complete removal of hair using a depilatory that (such as that available from Whitehall Laboratories, New York) is applied and left in contact with the skin for no more than 15 min. It must be washed away completely with a stream of warm running water. The animals are dried with a towel and the inside of the cages wiped clean of any depilatory before returning the guinea pigs.

When required, the epidermis is partially removed by tape stripping. The skin must be completely dry or the stripping will be ineffective. A length of tape approximately 8 in. long is used. Starting at one end of the tape, it is placed against the skin and rubbed with the finger a few times to cause good adhesion. It is then peeled away, taking with it some dry epidermal cells. A new section of the tape is then applied to the skin and the procedure repeated four or five times. The skin will have a shiny appearance owing to the leakage of moisture from the dermis. The tape should not be jerked away from the skin as this can cause the rupture of dermal capillaries.

The potential of the animal to respond to a sensitizer is enhanced by the injection of FCA (Calbiochem, San Diego, California, or Difco, Detroit, Michigan). The adjuvant is diluted 1:1 with sterile water before using. The injections must be id. In the Armstrong assay, a pattern of four

0.1 mL injections are given just prior to the first induction patching in the nuchal area. All four injections should fit under the edge of the area to be covered by the Hill Top Chamber. It is advisable to perform the skin-stripping operation before the injections, since adjuvant can leak onto the skin and prevent effective removal of the epidermis.

The occlusion of the patches is done in the same manner as described for the Buehler assay. The test site(s) is exposed to the UVA light after 2 hours of occlusion. The animal is left in the restrainer and the dental dam above the test site to be exposed is cut and the patch removed. Sites not to be exposed are left patched. Excess material is wiped from the site to be exposed, and the remaining parts of the animal are covered with aluminum foil. All patches are removed after the light-exposure step, and patched areas wiped free of excess material, and the animal returned to its cage.

Grading — The grading is the same as used in the Buehler assay.

Vehicles — With the exception of water, it is desirable to use a vehicle for the inductions that is different from the one used at the challenge (see Buehler assay). Since the control animals in the Armstrong assay are sham treated (including any vehicle), one can patch the test and control animals with vehicle at the time of challenge if the same vehicle must be used for both the induction and the challenge. It is advantageous to use a vehicle that dissolves the test material, though suspensions may not be avoidable in all cases.

Irritation Screens — The irritation screen is used to determine acceptable concentrations for the induction phase (i.e., one that does not produce eschar with repeated exposure or systemic toxicity) and the challenge phase (no more than slightly irritating). Each concentration must be tested with and without exposure to UVA light, as both conditions are used in the challenge. Thus, to evaluate four concentrations requires that eight animals be used. Each animal receives a pair of patches, with each pair being a different concentration (i.e., each concentration is patched on four animals). One of each pair of patches is placed on the left side and the corresponding concentration on the remaining patch is placed on the right side. The hair is removed by depilation the day of patching. The patches on the right side are removed after 2 hours of occlusion, the remaining parts of the animal covered with foil, and the right side exposed to 10 J/cm^2 of UVA light. Animals are returned to their cages after the exposure. If different solvents are being used in the induction and challenge phase, then two separate screens need to be run.

Conducting the Armstrong Assay — Combining the discussed techniques in a specific regimen yields the assay as follows:

1. Irritation/toxicity pretest (eight animals)
    a. Day 0: Remove the hair from the lumbar region by clipping and depilation. Apply to concentration on each animal on adjacent left side/right side locations for a total of four dose concentrations. Occlude the patches for 2 hours (±15 min). Expose the right side to 10/cm^2 of UVA light after removing the patches on the right side. Remove the remaining patches and excess material after the exposure to light.
    b. Day 1: Grade all test sites 24 hours (±1 hour) after removal of all patches (24-hour grade).
    c. Day 2: Repeat the grading 48 hours (+2 hours) after removing the patches (48-hour grade).
2. Induction (20 test + 10 sham controls + any rechallenge controls)
    a. Day 0: Weigh all test and control animals. Remove the hair from the mocha area with clippers and depilatory. Remove the epidermis by stripping four to five times with tape. Make four 0.01 mL id injections of a 1:1 dilution of ICA in an area to be covered by the patch. Cover this area on the test animals with a Hill Top Chamber that has 0.3 mL of test material preparation

in it. Patch the sham controls with water or solvent on the patch. Occlude with dental dam and restrain in a holder for 2 hours (±15 min). Remove the patches, cover the nonpatched areas with foil, and exposure to 10/cm^2 of UVA light for 30 min.

b. Days 2, 4, 7, 9, 11: Repeat the activity of day 0 with the following exceptions: Do not weigh animals and do not inject adjuvant. Move the patch back when the original induction site becomes too damaged but remain in the nuchal area. Depilation may not be needed at each induction.

3. Challenge (20 test + 10 sham control animals 9–13 days after last induction exposure)

a. Day 0: Weigh all animals; clip the lumber region free of hair and depilate. Do not strip the skin. Patch each animal with a pair of adjacent patches (one on the left side and one on the right side) containing 0.3 mL of a nonirritating concentration of test material in a Hill Top Chamber. Occlude the patches and restrain the animal for 2 hours (±15 min). Remove the patches from the right side and cover the rest of the animal's body with foil. Expose the right side to 10 J/cm^2 of UVA light. Remove the remaining patch and any excess material.

b. Day 1: Grade all challenge sites, keeping separate the grades of the site exposed to light and those not exposed to light 24 hours (±1 hour) after removal of the patches (24-hour grade).

c. Day 2: Repeat the grading 48 hours (±2 hours) after removal of the patches (48-hour grade).

4. Rechallenge

All or selected animals may be rechallenged with the same or a different test material 7–12 days after the challenge. Use 10 new sham-treated controls and naive test sites on all animals following the same procedure as used in the challenge.

5. Interpretation of results

Determine the number of positive responders (number of animals with a score >1 at either the 24 or 48 hours grading or with a score 1 unit higher than the highest score in the control). Determine the average score at 24 hours and at 48 hours for the test and control groups using face values. Keep the data for the sites exposed to light separate from the data from sites not exposed to light.

Strengths and Weaknesses — The Armstrong assay was found to give responses in the guinea pig that were consistent with what has been observed in humans: positive responses for 6-methylcoumarin and musk ambrette. One major disadvantage is that the procedure is time consuming with six induction exposures; additional work might demonstrate that fewer exposures will yield the same results.

The procedure is very stressful on the animals because of the injection of adjuvant and the multiple skin strippings and depilation.

As with any assay involving the intradermal injection of adjuvant, there is often a problem with using the results of the irritation screen in naive animals to accurately predict the results that will be seen in the sham controls at the challenge. If the material being tested is a nonirritant, or if one selects a concentration of an irritant that is far below the irritating concentration, then the screen does an adequate job of predicting the background irritation level in the challenge controls. However, if a slightly irritant concentration of an irritant is used, then the screen often underpredicts the irritation response and a high background level of irritation is observed at the challenge in the sham controls. The interpretation of the results of the challenge becomes difficult. The use of animals in the irritation screen that have had a prior injection of adjuvant might provide a viable alternative and reduce the number of times that rechallenges must be run because of high background levels of irritation.

The Armstrong assay was designed to evaluate materials for their photoactivated sensitization potential and not their potential to be nonphotoactivated dermal sensitizers. At this time, there are no background data that will allow for proper positioning of results of the Armstrong assay with regard to human risk if the assay indicates that a test material is a sensitizer or that a material is both a sensitizer and a photoallergen. Thus, it is highly recommended that a "standard" sensitization assay that can be related to humans be run before or in conjunction with the photosensitization assay. The use of a subjective grading system can be a source of significant verification.

Host Resistance Test

The guinea pig can also be used for the host resistance assay, a primary immunotoxicity screen. As such, it also now sees significant use in screening for drugs for tuberculosis. A protocol for such an assay is as follows.

Test System

1. Male Hartley guinea pigs (Charles River Breeding Laboratories; Portage, Michigan) are used. The guinea pigs should be 7–8 weeks old at the time of dosing. Animals will be acclimatized for at least 14 days prior to dosing.
2. Animals that appear abnormal should be rejected prior to dosing or assignment to a test group.
3. Animals are randomly selected from the pool of available animals and assigned to each dosage group.
4. Each animal is identified by a cage card.

Challenge Organism

1. *Pseudomonas aeruginosa* culture, from American Type Culture Collection, 27853, is grown in tryptic soy broth. This broth will be centrifuged down, and the resulting pellet being washed and resuspended in 0.9% saline. Plate counts should be performed to determine the concentration of the bacteria; the solution may be adjusted to give the desired concentration. Once prepared, samples of the bacterial solution will be repeated to confirm the concentration of the bacteria.
2. Administration of the test organism is intravenously (iv), in order to cause septicemia. Based on experimental data, a dosage of 2.3CFIJ (colony-forming units) × 108/kg should be expected to challenge the immune system of the guinea pig and provide a minimally lethal response. Each animal should receive a single exposure. They should be lightly anesthetized with ether and the inoculum should be administered into an ear vein. The dose volume should be 1 mL/kg of body weight.
3. The concentration (determined upon solution preparation) of the *P. aeruginosa* is confirmed by the replating of the samples taken after preparation.
4. Once prepared, *P. aeruginosa* cultures should be stored in a refrigerator for no more than 3 days.
5. All materials that come into contact with the organism should be placed in plastic bags, marked as a biohazard, and incinerated as soon as possible.

Study Design

Group	#/Group	Test Material (mg/kg/day)	*P. aeruginosa*
1	10	—	Yes
2	10	—	Yes
3	10	—	No

The guinea pig should be treated by the appropriate route with test material daily for 7 days. Guinea pigs should receive an iv dosage of *P. aeruginosa* on day 8 at a dosage of approximately 2.3CFU × 108/kg. The experiment is then terminated on day 15.

Observations

1. Prior to *P. aeruginosa* challenge, guinea pigs are observed at least twice daily. On the day of challenge, they should be observed periodically during the first 4 hours after challenge and then at least three times daily for the next 7 days for obvious signs of treatment, including death. Body weights of all animals will be determined prior to dosing on study days—3, 1, 8, and 15. Rectal temperatures are determined on days 1, 1 (2 hours postdosing), 2, 3, and 4, after *P. aeruginosa* challenge.

2. Animals found dead should be discarded. Necropsies are not conducted. Animals killed by design should be asphyxiated with carbon dioxide and discarded. Sacrifice schedule: Surviving animals are killed on study day 15 (7 days after dosing of *P. aeruginosa*).

Analysis and Interpretation

1. Mortality data will be analyzed by probit analysis or minimum normit chi-square analysis. Means and standard deviations are prepared for survival time body weights and rectal temperatures. Time-to-death analysis is also done.
2. A significant increase in mortality should be interpreted as a positive finding in this test system.
3. Diet can modify results (McFardland et al., 2008).

Occular and ototoxicity of the guinea pig also provides the most common traditional models for cataractagenesis and ototoxicity and is frequently used as a follow-up or investigative toxicity model for these (Ernstrom, 1970).

PATHOLOGY

John Peckham

Usefulness: Strengths and Weaknesses

Respiratory System

The sensitivity of the respiratory system of the guinea pig has provided animal models for numerous studies of inhalation phenomena, including bronchospasms, asthma, other respiratory allergies, hypersensitivity reactions to dust and other air pollutants, and development of antihistamines.

Digestive System

Guinea pigs are fastidious eaters. As they mature, they develop rigid habit patterns that must be accommodated if the animal is to thrive. Any changes in feed (taste, odor, texture, and form), water, feeder, or watering device may cause the guinea pig to stop eating or drinking (Harkness and Wagner, 1983). Starvation with clinical or subclinical illness from vitamin deficiencies may result. The intestinal tracts are sensitive to enterotoxins such as colchine (Yamada et al., 2000) and enterosiderosis (Takahashi et al., 1988).

The behavior of coprophagy and anatomical structure of a glandular stomach with a large cecum may modify the effects of experiments involving nutritional factors (Navia and Hunt, 1976).

Urogenital System and Fetal Tissues

Although the guinea pig has been employed infrequently in the past, it is unique among the animals available for reproductive and teratological studies. It occupies a position intermediate between the laboratory rodents, rats and mice, and the more evolved subhuman primates and man. For example, the endocrine control of its reproduction is similar to that of humans, even to the trimesteric characteristics, yet pregnancy is preceded by a well-defined estrus. Its placenta appears to be capable of endocrine activity, but it is labyrinthine, and its transfer functions are supplemented by an everted yolk sac exposed to uterine secretions. The elements of the reproductive cycle that precede pregnancy, i.e., estrus, ovulation, and fertilization, can be accurately determined as with all rodents, but its relatively long gestation allows the assessment of the effect of potentially harmful agents applied late in development upon organ functions or behavior patterns that develop after birth

in other rodents (Hoar, 1976b,c). Their long gestation period of 68 days provides an opportunity for separating toxic or teratogenic effects on the embryo from those upon the fetus and allows investigation of a fetus with an essentially mature central nervous system prior to delivery (Hoar, 1976b). In many instances, pregnancy is maintained following ovariectomy at 25 or more days. The guinea pig is, therefore, an animal of choice for studying the effects of hormones and endocrine glands on pregnancy (Harkness and Wagner, 1983). The circulatory form of pregnancy toxemia in guinea pigs has been identified as a possible model for preeclampsia in women (Percy and Barthold, 2001).

Malformations have been induced in guinea pigs by a variety of agents, including drugs, dyes, pesticides, vitamin excess, hyperthermia, adrenocortical hormone excess, synthetic steroids, ionizing radiation, and mineral deficient diets. Spontaneous malformations of genetic and unknown origin also occur (Hoar, 1976b,c).

Nervous System and Special Sense Organs

Because of the anatomy of the guinea pig ear and associated structures, it is an important animal model for ototoxicity studies. A postauricular surgical approach to the middle ear presents no major blood vessels or muscles. The petrous bone (otic capsule) is easily entered and dissected away without drilling to expose the inner ear structures in much less time than the cat and monkey. The guinea pig has been used to elucidate the pathophysiology of drugs that cause deafness and vestibular disorders, including the aminoglycosidic antibiotics such as neomycin, gentamicin, and other members of the streptomycin family, the diuretic ethacrynic acid, quinine, and salicylates (McCormick and Nuttall, 1976). In addition to ototoxicity, the guinea pig provides animal models for studies of noise, proprioception, Meniere's disease, amyotrophic lateral sclerosis, experimental allergic encephalomyelitis, spinal cord injury, epilepsy, multiple sclerosis, as well as development of antianxiety and anti-inflammatory compounds (Hanes, 2003).

Integumentary System and Soft Tissues

Guinea pigs have been used extensively for the study of immunological and inflammatory reactions involving the skin and subcutis. They are frequently used to test the safety of skin lotions and ointments. The dermal response of the guinea pig to irritation by test materials has been shown in many instances to be more like that of man than the rabbit; therefore, the guinea pig is the species of choice for dermal irritation tests (Gilman, 1982). In addition, physiological characteristics of its skin are similar to those of humans, which have led to other areas of investigation such as that of wound repair and thermal burns (Hoar, 1976a). Also, the guinea pig has provided animal models for studies of psoriasis and chemical burns such as mustard gas (Hanes, 2003).

The capability of chemical compounds to cause skin sensitization was first demonstrated in guinea pigs by Sulzberger in 1930, then Landsteiner and Jacobs (1935, 1936) and others. Using substituted benzene compounds, they demonstrated the development of a true allergic reaction following sensitization of the skin. Subsequently, experiments proved that skin could be sensitized by the administration of a chemical either topically, intradermally, or intraperitoneally. Skin sensitization tests are designed to determine if a chemical compound will cause an allergic reaction after there has been a previous contact to the same or a similar compound. The Hartley strain of guinea pig is the laboratory animal most commonly used for these tests (Gilman, 1982). Individual guinea pigs have been demonstrated to inherit differing skin susceptibilities to some compounds such as 2,4-dinitrochlorobenzene and poison ivy (Chase, 1941).

The female has one pair of inguinal mammary glands. Despite the apparent shortage of nipples, adult females have successfully raised litters of three, four, and more offspring (Harkness and Wagner, 1983). Abdominal mesothelioma has also been seen in aged animals exposed to asbestos (Wilson and Brigman, 1982).

Hematopoietic and Lymphoid Systems

The bone marrow of the guinea pig has been studied extensively because of its similarity to that of humans, its ease of dispersion to give uniform cell suspensions, and the ease of staining and identification of its cells (Sisk, 1976). Also, hormonally and immunologically, the guinea pig more nearly resembles the human than do rats and mice (Ernstom, 1970). Unlike many species that are very immature at birth, the neonatal guinea pig possesses very mature myeloid and lymphoid tissues. There is considerable data on cellular elements, physiological properties, and biochemical characteristics of circulating blood and bone marrow (Sisk, 1976). As a corticosteroid-resistant species, treatment with steroids does not markedly affect thymic physiology or peripheral lymphocyte counts (Sisk, 1976).

Mature females are an excellent source of serum complement used in a variety of immune and endotoxin serological reactions. Guinea pigs produce antibodies to specific proteins and are used to test for the presence or absence of small amounts of antigens by the production of anaphylaxis. Unlike the rabbit or chicken, injected antibodies protect the guinea pig from anaphylaxis (Harkness and Wagner, 1983). Delayed hypersensitivity can gave profound effects in the guinea pig, including massive hemorrhagic reactions (Stone, 1962).

Nutrition and Metabolic Diseases

The guinea pig is uniquely susceptible to vitamin C (ascorbic acid) deficiency, which results in bone/collagen disease and increased susceptibility to infections with inflammation, especially of the lungs and cervical lymph nodes. Streptozotocin effects on the pancreas make the male an attractive model for studying diabetes (Schlosser et al., 1984).

The vitamin C requirement makes the guinea pig a useful model for (1) nutritional studies pertaining to vitamin C metabolism, (2) collagen studies, (3) skin studies, (4) bone studies, (5) atherosclerosis studies, (6) adrenal–pituitary studies, and (7) hydroxylating reactions where ascorbic acid seems to play a role (Navia and Hunt, 1976). The guinea pig was used for the biological assay of vitamin C before the chemical assays were developed (Fenner, 1986).

Bacterial, Viral, and Rickettsial Diseases

Guinea pigs were once used extensively for disease diagnosis and isolation of pathogenic agents such as in tuberculosis (Jolly and Heywood, 1979). Guinea pigs are highly susceptible to both human- and bovine-type tubercle bacilli and have long filled an important place in tuberculosis research and diagnosis. A guinea pig model of low-dose aerogenic tubercular infections has been developed (Chambers et al., 2001). Guinea pigs infected with the Legionnaires' disease bacillus *Legionella pneumophila* have been used to evaluate new therapeutic agents (Edelstein et al., 2001, 2003). They are also susceptible to rickettsial infections, and a number of rickettsiae pathogenic to humans were first studied in the guinea pig (Fenner, 1986). Guinea pigs are susceptible to the Q fever agent *Coxiella burnetii* and provide an animal model of this disease (Heggers et al., 1975). The guinea pig provides animal models for the development of a wide variety of therapeutics including antibiotic, antifungal, and antiviral compounds (Hanes, 2003).

Neoplastic Diseases

Although guinea pigs have been used in large numbers as experimental laboratory animals, they have not been used as frequently in carcinogenesis studies. This has been in part because

of a prevailing view that guinea pigs are particularly resistant to induced neoplasia. This genetic susceptibility to spontaneous neoplasia appears to vary (Percy and Barthold, 2001).

Guinea pigs were considered to be resistant to chemical carcinogens until 1962 because exposure to chemicals known to be carcinogenic in rats failed to produce tumors (Mosinger, 1961; Argus, 1971). This observation led to investigation of tumor-resistant factors in guinea pigs. Guinea pig serum was found to have a factor that inhibited the growth of several lymphomas in mice. This factor was termed tumor inhibitory principle (TIP), but it could not be isolated. The existence of this principle is still uncertain (Manning, 1976). A serum factor (probably asparaginase) in normal guinea pig sera has been demonstrated to have antitumor activity. Splenic preparations containing large numbers of Kurloff cells have shown inhibition of transformed human epithelial cells in vitro (Percy and Barthold, 2001). Since 1962, guinea pigs have been shown to be susceptible to the carcinogenic activity of a variety of agents, including physical and chemical irritants, hydrocarbons, nitrosamines, and hormones, particularly estrogens (Torn, 1970; Manning, 1976).

The refractoriness of the guinea pig to the carcinogenic action of aromatic amines and amino azo dyes possibly resides in its limited ability to metabolize these agents to their *N*-hydroxy derivatives. Miller et al. (1964) showed that *N*-hydroxy-2-acetylaminofluorene in guinea pigs induced adenocarcinomas of the small intestine (upon feeding) and sarcomas (upon injection), whereas 2-acetylaminofluorene is inactive. Very rapid elimination of *N*-hydroxy derivatives from guinea pig tissues has been proposed as another mechanism (Kiese and Wiedemann, 1968). Berenblum (1949) found that increasing the dosage of 9,10-dimethyl-l,2-benzanthracene to 20-fold that of the rat resulted in tumors in guinea pigs. Rogers and Blumenthal (1960) induced tumors in 57% of 735 guinea pigs with methylcholanthrene injected subcutaneously and intramuscularly.

Guinea pigs are susceptible to many different chemical carcinogens, including ethyl carbamate, polycyclic hydrocarbons (7,12-dimethylbenz[*a*]anthracene, 3-methylcholanthrene, benzopyrene, 1,2,5,6-dibenzanthracene, and others), nitrosamine-type alkylating agents (diethylnitrosamine, dimethylnitrosamine, *N*-methyl-*N*-nitrosourea, N-nitroso-N-methyl-N-dodecyclamine, nitroso-2,6-dimethylmorpholine, dinitroso-2,6-dimethylpiperazine, di-*N*-butylnitrosamine), methylazoxymethanol, aflatoxin, and dioxan (Argus, 1971; Squire et al., 1978).

Animals used in carcinogenicity studies must be reasonable in cost to allow adequate numbers of both test and control animals, have a life span within both the financial capabilities of the sponsor to maintain them and the public health and political time constraints for determining an answer, and be well adapted to the laboratory environment without serious interfering infectious diseases. Although mice and rats meet the first two requirements better than guinea pigs, guinea pigs should be considered if the metabolism of the compound and its availability at the expected target site are similar to that in humans and if the value or extended use of the test product warrants the extra cost (Robens et al., 1982).

It is important for the testing laboratory to establish the background tumor incidence and other lesions of aging guinea pigs to help determine if the incidence of tumors among the controls in any specific test is representative. The laboratory incidence should be compared with reported tumor incidence when the latter are available (Robens et al., 1982).

ORGAN WEIGHTS AND RATIOS

Organ weights have rarely been reported by investigators because of the infrequency of guinea pigs to be used in acute, subchronic, and chronic toxicity tests by the chemical, cosmetic, and pharmaceutical industries. The following organ/body weights were calculated based on data reported by Breazile and Brown (1976).

Organ	Weight (g)	Organ/Body Weight Ratio[a] (× Adjustment Factor)		Organ/ Brain Ratio
Brain	4.300	4.778	(×1000)	—
Spinal cord	14.500	1.611	(×100)	3.372
Pituitary	0.022	0.024	(×1000)	0.005
Thyroid	0.134	0.149	(×1000)	0.031
Adrenal	0.725	0.806	(×1000)	0.169
Liver	42.500	4.722	(×100)	9.884
Lung	5.000	5.556	(×1000)	1.163
Kidneys	6.120	6.800	(×1000)	1.423
Pancreas	2.500	2.778	(×1000)	0.581
Testes	4.300	4.778	(×1000)	1.000
Epididymis	0.660	0.733	(×1000)	0.153
Ovaries	0.192	0.213	(×1000)	0.045
Stomach	0.420	0.467	(×1000)	0.098
Intestine	28.000	3.111	(×100)	6.512
Urinary bladder	4.250	4.722	(×1000)	0.988
Stomach and intestine (with contents)	120.00			
Eyes	1.250			
Skeleton and ligaments	64.000			
Musculature	320.00			

[a] Based on 900 g guinea pig.

For additional information, see the other portions of this chapter and see Turton et al. (1977a,b).

SPONTANEOUS LESIONS: NONNEOPLASTIC AND NEOPLASTIC

General Considerations: Nonneoplastic Findings

Guinea pigs are nervous in temperament and respond to sudden noises, unfamiliar surroundings, and sudden movements by freezing in place or a random stampede, which can result in injuries. They may refuse to eat or drink for some time following any significant change in location, feed, or other management practice. They require a constant source of water and must be trained to lick sipper tubes. Males and some females are prone to fight cage mates, which can result in severe injuries, even deaths. Their large size at birth contributes to dystocia. The major diseases affecting guinea pigs are scurvy, respiratory tract infections, and enteritis (Percy and Barthold, 2001).

Anorexia usually accompanied with weight loss can result from many conditions including changes in their housing or mechanical failure, water deprivation, unfavorable room temperature, changes in available food, unpalatable or contaminated food, malocclusion, oral lacerations, obesity, ketosis, renal failure, urolithiasis, vitamin C deficiency, protein deficiency, metastatic calcification, pododermatitis or infections at various other sites, neoplasia, ectoparasitism, loss of a cage mate, or antagonism of a dominant cage mate (Hanes, 2003).

Deaths may occur from excessive cold or heat, septicemia, toxemia, *Salmonella* infection, enteritis, pregnancy toxemia, antibiotic toxicity, pneumonia, volvulus of cecum or stomach, dystocia, dehydration, fractured limb, or being caught in cage floor (Hanes, 2003).

General Considerations: Neoplastic Findings

Considering only animals of comparative ages, guinea pigs have a lower incidence of spontaneous neoplasms than rats or mice (Morgan, 1969), particularly virus-free animals (Rhim et al., 1976).

Accurate estimates of the incidence of spontaneous neoplasia in any species must be based on complete necropsies and thorough microscopic examination of tissues from animals allowed to live one-half or more of the natural life span (Manning, 1976; Peckham, 1980). The breeding life of laboratory guinea pigs is from 18 months to 4 years, and they have been known to live 8 years; however, they rarely survive in the home longer than 5 years (Harkness and Wagner, 1983; Ruf, 1984). The life span of the laboratory guinea pig is 4–6 years (Wallach and Boever, 1983). An occasional animal may reach 9.0 or 9.5 years of age (Kunstyr and Naumann, 1984). There are few definitive reports in the literature that meet the criteria of complete necropsy examinations with histopathology and full life spans. Shimkin and Mider (1941) reported no neoplasms in 15,000 guinea pigs of inbred strains born and observed for up to 5 years between 1916 and 1937. Papanicolaou and Olcott (1942) observed about 100 tumors in over 7000 guinea pig necropsies for an incidence of 1.4%; tumors were rare in animals less than 4–5 years of age.

Rogers and Blumenthal (1960) examined 6000 guinea pigs of two inbred strains over a 10-year period. These animals, which had not been used for experimental purposes, had a spontaneous tumor incidence of 0.4% for all ages and 14.4% (14 of 97) for animals surviving 3 years or more. All 14 tumors occurred in the R9 strain. The incidence of tumors in random-bred Hartley strain guinea pigs from necropsies of 8400 animals (estimated male and female ratio 1:3) at ages of less than 27 months was 0.75% and of 34 retired breeders (6 males and 28 females) greater than 27 months of age was 29.4%.

Cardiovascular System

Generalized cardiovascular system septicemias sometimes occur with bacterial infections such as staphylococcosis and those caused by *P. aeruginosa*, *Pasteurella multocida*, and *P. haemolytica*. Pregnancy toxemia, a common problem in guinea pigs, has a circulatory form (Percy and Barthold, 2001). The guinea pig provides animal models for studies of implants, septic shock, vasoconstriction, and antiarrhythmic compounds (Hanes, 2003).

Heart

Nonneoplastic Lesions, Spontaneous

Cardiac "rhabdomyomas" also termed rhabdomyomatosis are commonly observed circumscribed accumulations of glycogen that might be confused with neoplastic lesions. Rhabdomyomatosis is considered to be an incidental finding resulting from a congenital disease in glycogen metabolism (Lehr, 1965; Vink, 1969; Manning, 1976; Hoch-Ligeti et al., 1986; Percy and Barthold, 2001).

Spontaneous interstitial lymphocytic myocarditis of unknown pathogenesis has been observed as an incidental finding in guinea pigs that is similar to that described in mice, rats, and rabbits (Miller, 1924). Vegetative endocarditis can be caused by *Streptococcus* spp. infections. Minoxidil can cause atrial pathology in man and other animals (especially the guinea pig; Sobota et al., 1980). The guinea pig is also a good screening model for rhabdomyomatosis (Takahashi and Iwata, 1985).

A colony of guinea pigs with myocardial necrosis and mineralization was investigated for the possible roles of vitamin E or selenium deficiencies. Vitamin E and selenium levels were within normal ranges suggesting that these heart lesions resulted from inbreeding within the colony (Griffith and Lang, 1987).

Neoplastic Lesions, Spontaneous

Spontaneous cardiovascular tumors are uncommon. Benign mesenchymal mixed tumors (mesenchymomas or myxomas) have been observed in the heart of 12 females of the Hartley strain (McConnell

and Ediger, 1968; Manning, 1976). These tumors included well-differentiated mesenchymal components, such as cartilage, bone, and fat. They should not be confused with rhabdomyomatosis, congenital glycogen lesions (Percy and Barthold, 2001). Also, a fibrosarcoma of the heart and a cavernous hemangioma of the liver have been reported (Rogers and Blumenthal, 1960; Manning, 1976).

Aorta and Arteries

Nonneoplastic Lesions, Spontaneous. The circulatory form of pregnancy toxemia in guinea pigs is characterized by uteroplacental ischemia due to compression of the aorta caudal to the renal blood vessels by the gravid uterus. This results in reduced blood pressure in the uterine vessels and tissue anoxia, with subsequent placental necrosis, uterine hemorrhage, thrombocytopenia, ketosis, and death. The disease has been considered a possible model for preeclampsia in women (Percy and Barthold, 2001).

Nonneoplastic Lesions, Induced. Medial calcification of the major elastic arteries and soft tissue calcification have been associated with hypervitaminosis D (Innes, 1965; Wallach and Boever, 1983; Perfumo et al., 1999).

Respiratory System

Lung

General Considerations—Clinical respiratory signs can result from bacterial or viral pneumonia, heat stress, diaphragmatic hernia, pregnancy toxemia, or gastric torsion (Hanes, 2003).

Nonneoplastic Lesions, Spontaneous

Susceptibility to acute anaphylaxis is related to the quantity of histamine available for release in the lung. In highly susceptible Hartley animals, this amount can be more than 10 times greater than in the resistant strain 2. Strain 2 and Hartley guinea pigs are equally susceptible to histamine toxicity (Stone et al., 1964).

Adenomatosis or alveolar epithelial hyperplasia is a frequent lung lesion in guinea pigs that must be differentiated from alveolar or bronchogenic adenomas (Hoch-Ligeti et al., 1982). Proliferations of alveolar epithelium diagnosed as tumors were often associated with interstitial pneumonia, foreign bodies, or other inflammatory changes, which suggest that many of these lesions are in reality hyperplasia (Manning, 1976).

Pneumonia is caused by one of several bacteria or viruses in guinea pigs. The most important of these bacteria include *Bordetella bronchiseptica*, *Klebsiella pneumoniae*, *P. multocida* or *P. pneumotropica*, and *Streptococcus* (*Diplococcus*) *pneumoniae* (Saito et al., 1983). Other microorganisms associated with pneumonia in guinea pigs are *Streptobacillus moniliformis* (Kirchner et al., 1992), *Corynebacterium kutscheri*, other *Streptococcus* spp., *P. aeruginosa*, *Citrobacter freundii* (Ocholi et al., 1988), and *Mycoplasma pulmonis*. Signs in affected guinea pigs include rough hair coat, anorexia, emaciation, hunched posture, dyspnea, abdominal breathing, rales, sneezing, and sometimes a mucosanguineous to purulent discharge from the eyes and nose. At necropsy, *B. bronchiseptica* has been shown to cause partial consolidation of the lungs. Histologically, a marked purulent bronchitis and bronchiolitis or fibrinous bronchopneumonia can be observed. *S. pneumoniae* commonly causes a fibrinopurulent pleuritis or peritonitis (Parker et al., 1977). *Streptococcus zooepidemicus* may cause septicemia and pneumonia. *P. aeruginosa* may cause a focal necrotizing and granulomatous pneumonia with "sulfur" granules or green exudate. Pneumonias caused by *Escherichia coli* and *K. pneumoniae* are usually secondary and accompanied by pleuritis, pericarditis, and peritonitis (Ganaway, 1976; Wagner, 1979; Wallach and Boever, 1983; Ruf, 1984).

Guinea pigs that are latent carriers of *S.* (*Diplococcus*) *pneumoniae* frequently die of fibrinopurulent peritonitis, pericarditis, pleuritis, pneumonia, or meningitis following the injection of irritating substances into or removal of fluids from body cavities (Wagner, 1976). Vitamin C deficiency results in an increased susceptibility to pneumonia and pleuritis from bacterial infections.

Adenoviral pneumonia was fatal to 11 of 1600 preadult guinea pigs. The only clinical sign was dyspnea. Grossly partial consolidation of the anterior lobes was observed in the lungs. Lesions were limited to the respiratory system, and included emphysema, petechiation, hydrothorax, bronchiolitis, bronchial epithelial sloughing, and focal parenchymal necrosis and inflammation. The desquamated bronchial epithelium frequently contained large basophilic intranuclear inclusion bodies. Numerous viral particles of an adenovirus were found (Naumann et al., 1981; Brennecke et al., 1983; Kaup et al., 1984; Kunstyr et al., 1984; Richter, 1986). Adenoviral infections are probably more prevalent than currently recognized. Frequently, outbreaks have occurred in animals subjected to experimental procedures that may have resulted in impaired immunity (Percy and Barthold, 2001).

Adiaspiromycosis caused by *Emmonsia parva* and *Emmonsia crescens* has been reported in guinea pigs. It is a benign self-limiting granulomatous pulmonary disease (Wallach and Boever, 1983).

Perivascular lymphoid nodules in the lungs of guinea pigs occur around smaller branches of the pulmonary arteries and veins. They have been reported as occurring in 14%–85% of guinea pigs of both sexes and all ages from seven strains, including a germfree animal (Thompson et al., 1962). The nodules appear to enlarge with age and may be visible grossly as pinpoint, subpleural foci. These normal lymphoid nodules must be differentiated from focal granulomatous pulmonary lesions seen after treatment with Freund's adjuvant (Percy and Barthold, 2001).

Pulmonary arteries and arterioles are greatly thickened as a result of prominent smooth muscle layers in the tunica media. This is not an abnormal finding (Percy and Barthold, 2001).

Nonneoplastic Lesions, Induced

Histamine administration results in lethal bronchiolar smooth muscle contraction. When prostaglandin IR-PGF20 generation was studied, the peripheral lung responses closely resembled those of the peripheral human lung to histamine H1 stimulation (Steel et al., 1979).

Experimental airborne tuberculosis in the guinea pig provides a useful model of human tuberculosis with a naturally occurring bacillemia and cavitary stages of the disease (Smith and Harding, 1977). A guinea pig model of low-dose, aerogenic tubercular infections may be suitable for virulence, vaccination, and immunological studies (Chambers et al., 2001).

Induction of pneumonic pasteurellosis in guinea pigs provides a model for studying bovine pneumonic pasteurellosis, shipping fever pneumonia (Morck et al., 1990).

Eosinophilic granulomas were observed in the lungs as a result of visceral larval migrans due to *Baylisascaris procyonis* eggs in wood shavings bedding contaminated by raccoon feces (Van Andel et al., 1995).

Pneumoconioses (foreign body pneumonias) are incidental findings in the lungs, especially in young guinea pigs. They are associated with aspirated food or bedding materials. They have been observed as foci or circumscribed nodules of granulomatous or chronic active inflammation in the bronchioles or alveoli with mononuclear cells and foreign body giant cells. Plant fibers or other foreign materials may be identified. These findings may complicate respiratory research studies. Pulmonary multifocal granulomas may also occur following subcutaneous injection with Freund's adjuvant (Percy and Barthold, 2001).

Bony spicules (osseous metaplasia) are occasionally observed in the lung. They consist of dense lamellar bone, with varying degrees of calcification and sometimes include well-differentiated bone marrow. They have been observed in the interstitium of alveolar septa of the lungs of guinea pigs fed commercial diets (Kaufmann, 1970). Large numbers of these metaplastic osseous foci have been observed following x-irradiation (Percy and Barthold, 2001).

Neoplastic Lesions, Spontaneous

Spontaneous lung tumors constitute some of the most important tumor types in guinea pigs and have been the most frequently reported solid tumors, approximately 35% in one survey. Most of the tumors were benign papillary adenomas and appeared to have bronchogenic origin (Percy and Barthold, 2001). Other reports include a number of alveologenic tumors, an adenoma, papillary or bronchogenic adenomas, and bronchogenic adenocarcinomas (Manning, 1976) as well as hemangiosarcoma, lymphangioma, and intrabronchial papilloma (Hoch-Ligeti et al., 1982).

Rogers and Blumenthal (1960) found that 64 papillary adenomas (2.6%) and 1 adenocarcinoma of the lungs had been reported in the literature. Eighteen adenomas and seventeen adenocarcinomas were reported by Mosinger (1961). These bronchoalveolar or alveologenic tumors are common in animals over 3 years of age. In some studies, the proliferations of alveolar epithelium that were considered to be tumors were associated with interstitial pneumonia, foreign bodies, or other inflammatory changes, which suggest that some of these lesions were hyperplastic, not neoplastic (Manning, 1976). Primary malignant tumors of the lung are rarely observed (Percy and Barthold, 2001).

Neoplastic Lesions, Induced

Irradiation resulted in the earlier occurrence of alveologenic tumors and an increased number of tumor nodules than in untreated controls. The number of alveologenic tumors in guinea pigs that survived over 20 months was not statistically significantly different between irradiated and untreated groups. The tumors observed in the lungs were alveologenic tumors (synonyms: papillary adenomas, alveolar adenomas, adenomas), hemangiosarcomas, lymphangiomas, intrabronchial papillomas, and adenomatosis (Hoch-Ligeti et al., 1982; Hoch-Ligeti and Argus, 1970).

Of 111 strains 2 and 13 guinea pigs injected intravenously with either 20-methylcholanthrene or 1,2,5,6-dibenzathracene, pulmonary tumors occurred in 32 animals with a similar frequency in both strains (Heston and Deringer, 1952).

Digestive System

Guinea pigs have a long colon (60% of the length of the small intestine vs. only 16% in the rat) with a characteristic large, thin-walled cecum with numerous lateral pouches that occupy the left side of the abdominal cavity. The taenia coli have served as a preferred source of smooth muscle for physiologists (Breazile and Brown, 1976).

Teeth

Nonneoplastic Lesions, Spontaneous

Malocclusion and loss of opposing teeth lead to the overgrowth of the teeth. Since all guinea pig teeth are open rooted and erupt continuously, all teeth have a tendency to overgrow, especially the premolars or the anterior cheek teeth (Ediger et al., 1975; Harkness and Wagner, 1983). Calcium deficiencies can cause soft maxillary and mandibular bone with misalignment of the teeth and overgrowth of the incisors and molars. Soft foods aggravate the problem of malocclusion. Clinical signs include excessive salivation (slobbers), loss of weight (wasting), anorexia (the animal is usually hungry but cannot eat), licking movements, and loss of the ability to close the mouth (Wallach and Boever, 1983). Dental abnormalities also occur with scurvy and exposure to fluorides (Percy and Barthold, 2001). In addition to improper diet, a genetic predisposition involving more than one gene has been reported in a high incidence in strain 13. Excessive salivation also can occur with heat stress (Hanes, 2003).

Salivary Gland

Nonneoplastic Lesions, Spontaneous. Cytomegalovirus is a well-described salivary gland disease of guinea pigs. It is a herpesvirus infection characterized by eosinophilic intranuclear and rarely by intracytoplasmic inclusion bodies in ductal epithelium of the salivary glands and the epithelium of the proximal and distal convoluted tubules of the kidneys. In the salivary glands, affected cells are markedly enlarged (Cook, 1958; Van Hoosier and Robinette, 1976; Kinkier et al., 1976; Percy and Barthold, 2001). Disseminated cytomegalovirus disease has been reported with involvement of the spleen, kidney, liver, and lung (Van Hoosier et al., 1985). The experimental disease is considered a useful animal model for infections in other species, including human patients (Percy and Barthold, 2001).

Neoplastic Lesions, Spontaneous. A benign mixed tumor of a sublingual salivary gland was reported in a 1.5-year-old guinea pig (Koestner and Buerger, 1965).

Esophagus

Neoplastic Lesions, Induced. Spontaneous tumors of the esophagus are apparently extremely rare; however, a squamous cell papilloma and carcinomas were produced by methylcholanthrene and nitrosopiperidine, respectively (Squire et al., 1978).

Stomach

Nonneoplastic Lesions, Spontaneous. Gastric bloat occurs in guinea pigs when excesses of fresh green forage are fed. The clinical signs of bloat include distended abdomen, abdominal pain, depression, and cyanosis (Wallach and Boever, 1983). Acute gastric dilatation associated with gastric volvulus was observed in multiple animals of one colony. Frequently affected animals were found dead without previous signs of illness (Lee et al., 1977). Gastric ulcers are fairly common; most are secondary to other diseases, especially ketosis (Jervis et al., 1973; Wagner, 1979; Ishihara et al., 1983).

Neoplastic Lesions, Spontaneous. Papanicolaou and Olcott (1940, 1942) reported five benign mesenchymal neoplasms in the stomach of guinea pigs. The tumors were a fibromyoma, three leiomyomas, and a lipoma.

Neoplastic Lesions, Induced. Adenocarcinomas and sarcomas of the glandular stomach were produced by methylcholanthrene (Squire et al., 1978).

Intestine

Nonneoplastic Lesions, Spontaneous

Guinea pigs are particularly prone to acute enteritis although peracute, subacute, and chronic forms also occur. Diarrhea may or may not be present. Nonspecific signs include anorexia, weight loss, depression, and conjunctivitis. Latent forms of the disease may be precipitated by stressful environment changes such as shipping, pregnancy, and research procedures. Acute enteritis has been also associated with subclinical scurvy (Clarke et al., 1980). One can perform endoscopy on guinea pigs to evaluate such lower intestinal tract lesions (Perez et al., 1980).

A wasting syndrome associated with viral enteritis has been reported in young guinea pigs following their arrival at a research facility. This syndrome is characterized by wasting, anorexia, and diarrhea. It has a low morbidity and mortality (Jaax et al., 1990; Marshall and Doultree, 1996).

Enterocolitis, acute necrotic cecitis, or typhlocolitis is a highly lethal disease that resembles antibiotic "toxicity." It is characterized by sudden death and acute necrosis of the intestinal mucosa and adjacent submucosa, especially in the cecum. This disease occurs sporadically in guinea pigs of all ages. No specific cause or causes have been identified. A thermolabile toxin has been found in some animals (Wagner, 1976, 1979). *Clostridium perfringens* has been isolated from some affected

guinea pigs. Also in other affected animals, *Clostridium difficile* has been demonstrated in the absence of prior antibiotic treatment (Percy and Barthold, 2001).

Among the most common forms of enteritis are those of salmonellosis and the fatal necrolytic enterocolitis. The *Salmonella* spp. most commonly found are *Salmonella typhimurium* and *Salmonella enteritidis*; however, other salmonellae including *Salmonella dublin* and *Salmonella limete* have been isolated. Other bacteria associated with enteritis include pseudotuberculosis caused by *Yersinia (Pasteurella) pseudotuberculosis* and *Yersinia enterocolitica*; colibacillosis caused by *E. coli*, *K. pneumoniae*, *C. freundii* (Ocholi et al., 1988), and *Arizona* spp.; Tyzzer's disease caused by *Clostridium piliforme* (formerly *Bacillus piliformis*) (Waggie et al., 1986); and *Pseudomonas* spp. Typical findings in cavian Tyzzer's disease are necrotizing ileitis and typhlitis that may be accompanied by characteristic focal coagulative necrosis of liver (Percy and Barthold, 2001). Common necropsy findings of salmonellosis include an enlarged red spleen, sometimes with pale foci of necrosis; enlarged liver with necrotic foci; enlarged lymph nodes; and congested intestines (Habermann and Williams, 1958; Wagner, 1979; Wallach and Boever, 1983; Jayasheela et al., 1985; John et al., 1988). In a young guinea pig with Tyzzer's disease, the *C. piliforme* bacilli were associated with cryptosporidiosis and unclassified intestinal spirochetes (McLeod et al., 1977).

Segmental duodenal hyperplasia associated with proliferation of intracellular bacteria was observed in a female guinea pig, 1 of 140 obtained from a commercial colony. Two cage mates died with acute enteritis without the mucosal hyperplasia (Elwell et al., 1981). The hyperplastic lesions have been attributed to *Lawsonia (Campylobacter) intracellularis* infections. These lesions and bacteria were associated with a spontaneous outbreak of diarrhea with weight loss and mortalities. Also, the disease has been observed during steroid treatment (Percy and Barthold, 2001).

Although a large number of protozoan parasites are observed in guinea pigs, protozoan diseases are rare. The most important protozoan disease is coccidiosis, which is caused by *Eimeria caviae*. Coccidia are sometimes seen in histological sections of the cecum and colon but rarely cause clinical disease. The clinical signs are anorexia, diarrhea, emaciation, dry hair coat, and rarely death. *Cryptosporidium wrairi*, a small coccidium of the small intestine, may be associated with chronic enteritis, diarrhea, weight loss, and emaciation. Clinical infections are observed most frequently in juvenile animals. Infection rates of 30%–40% are common in conventional colonies. Cryptosporidiosis is often accompanied by *E. coli* infections (Jervis et al., 1966; Percy and Barthold, 2001). Numerous other protozoa, including *Balantidium coli*, have been observed in the intestinal tract of guinea pigs with little evidence of clinical disease (Vetterling, 1976; Wagner, 1979).

The only intestinal nematodiasis reported in guinea pigs with any degree of frequency is the cecal worm *Paraspidodera uncinata*. It rarely causes clinical disease (Westcott, 1976; Wagner, 1979). Infections can result in diarrhea, lethargy, and anorexia (Wallach and Boever, 1983). No lesions are associated with these infections (Habermann and Williams, 1958).

Cecal torsion occasionally results in guinea pig deaths. In affected animals, the twisted cecum is edematous, hemorrhagic, and distended with fluid and gas (Percy and Barthold, 2001).

Germfree animals have disproportionately large cecum. The cecum is increased from about 10% in a conventional animal to 25%–30% of the body weight in germfree animals. This large cecum predisposes animals to cecal ruptures, herniation, torsion, volvulus, and uterine prolapse (Hanes, 2003).

Prolapse of the colon into the rectum resulting from colon intussusceptions is associated with stress, dehydration, and excessive straining (Hanes, 2003).

Intestinal hemosiderosis is the result of accumulations of hemosiderin-laden macrophages in the lamina propria of the intestine, particularly the large intestine. This is a common finding in guinea pigs. The cause is unknown but is suggested to arise from the normally zealous iron binding of herbivores, with uptake of excessive dietary iron (Percy and Barthold, 2001).

Nonneoplastic Lesions, Induced

Antibiotics should be used with caution. The abrupt changes in intestinal microflora following the use of several antibiotics, especially those with antibacterial activity against gram-positive organisms, cause high mortality rates from enteritis and what is thought to be endotoxic shock due to the overgrowth of gram-negative bacteria, with their accompanying toxins, in the large intestine (Wagner, 1979; Wallach and Boever, 1983). Other reports suggest a role of gram-positive *C. difficile*, in this syndrome (Percy and Barthold, 2001). Penicillin "toxicity" is a well-recognized fatal disease in guinea pigs. Other antibiotics, including bacitracin, chlortetracycline, oxytetracycline, streptomycin, aureomycin, lincomycin, methicillin, erythromycin, and chloromycetin, also have been reported to be toxic to guinea pigs. Penicillin was not toxic for germfree guinea pigs. Profuse diarrhea with high mortality can develop in up to 50% or more of the guinea pigs within 1–5 days following the antibiotic treatment. Enterotoxins of *C. difficile* have been demonstrated after treatment with penicillin (Percy and Barthold, 2001). The toxicity of intramuscular injections of chloromycetin was attributed to glycols used as vehicles (Altemeier et al., 1950; Hoar, 1976b).

Hartley guinea pigs infected with *Entamoeba histolytica* provide an animal model for enteric amebiasis, with the same type flora found in the original human host. The infection was produced by introducing *E. histolytica* trophozoites and the accompanying enteric flora obtained from humans into germfree guinea pigs (Jervis and Takeuchi, 1979).

Inflammatory bowel disease (ulcerative colitis and Crohn's disease) can be induced by feeding degraded carrageenan (Anver and Cohen, 1976 ; Duhamel, 2001), or by immunization with an initial sensitization by dinitrochlorobenzene followed with multiple intrarectal instillation of this same chemical (Rabin, 1980). Grossly, multiple pinpoint ulcers and hemorrhage were present in the cecal and colonic mucosae. Cecal and colonic lymphoid tissues were enlarged. Microscopically, the colonic and cecal mucosae have multiple crypt abscesses superficial mucosal ulcerations, depletion of mucus in cells lining the crypts, edema infiltration of the lamina propria by a variety of inflammatory cells, mucosal distortion craterous ulcers, and mucosal and submucosal granulation tissue resembling features of the human ulcerative disease (Anver and Cohen, 1976; Rabin, 1980; Moto et al., 1983).

Neoplastic Lesions, Spontaneous

Spontaneous tumors of the intestine are apparently infrequent. Two tumors have been reported, a fibroid and a liposarcoma of the intestine (Rogers and Blumenthal, 1960; Manning, 1976).

Abdominal Cavity

Nonneoplastic Lesions, Spontaneous. Abscesses in the visceral organs and peritonitis have been observed with staphylococcosis, *Pseudomonas* spp. (green exudate), and *Corynebacterium* spp. infections including *Corynebacterium pyogenes*, *C. kutscheri*, and *Corynebacterium pseudotuberculosis* (grayish and caseous exudate). Ultrasonography provides a new method for the diagnosis of abdominal diseases including abscesses (Beregi et al., 1999, 2000a,b).

Neoplastic Lesions, Spontaneous. One tumor, a fibrosarcoma, was observed in the peritoneal cavity (Manning, 1976).

Pancreas

Nonneoplastic Lesions, Spontaneous. The portion of exocrine pancreas decreases, whereas the portion of fat increases with age. There is no apparent impairment of function. Histologically, large areas of adipose tissue separate normal appearing pancreatic acini and ducts (Wagner, 1976; Hanes, 2003).

Neoplastic Lesions, Spontaneous. Adenomas or nodules of acinar cells are frequently observed in older animals (Squire et al., 1978).

Liver and Gallbladder

Nonneoplastic Lesions, Spontaneous

Multifocal hepatic necrosis and enteritis occur together in a variety of conditions. These include salmonellosis, Tyzzer's disease (*C. piliforme* formerly *B. piliformis*), pseudotuberculosis (*Y. pseudotuberculosis* and *Y. enterocolitica*), listeriosis (*Listeria monocytogenes*), and toxoplasmosis (*Toxoplasma gondii*) (Sparrow and Naylor, 1978; Zwicker et al., 1978; Boot and Walvoort, 1984). Hepatic multifocal necrosis is often accompanied by similar lesions in the spleen. Also, hepatic necrosis has been observed after infections by cytomegalovirus and possibly other herpes-like viruses (Percy and Barthold, 2001). Multifocal coagulation necrosis in livers is occasionally seen at necropsy. Affected areas are usually subcapsular with minimal or no inflammation suggesting that they arise as terminal events and secondary to localized impaired blood flow (Percy and Barthold, 2001). Similar focal lesions were reported in clinically normal guinea pigs (Cuba-caparo et al., 1977) and have been produced by repeated administration of halothane (Hughes and Lang, 1972). A marked fatty liver often is associated with ketosis.

Hemorrhage into the peritoneal cavity from tears of the liver capsule is occasionally observed at necropsy. These traumatic lesions may be caused by falls or mishandling (Percy and Barthold, 2001).

Periportal fibrosis associated with chronic hepatopathy occurs occasionally in adult guinea pigs as an enzootic problem. The lesions are usually concentrated around portal triads and characterized by hepatocyte degeneration, proliferation of cholangioles, and interstitial fibrosis. These findings are suggestive of a toxin-induced change (Percy and Barthold, 2001). Adults also spontaneously for gallstones (Gurll and Denbesten, 1979).

Nonneoplastic Lesions, Induced

Liver lesions have been produced by ingestion of pyrrolizidine alkaloids in *Crotalaria spectabilis* seeds. Clinical signs include ascites, distended abdomen, hepatic necrosis, thrombosis of portal veins, and focal hepatic fibrosis (Carlton, 1967; Chesney and Alien, 1973).

Neoplastic Lesions, Spontaneous

Spontaneous hepatobiliary neoplasms in the guinea pig are very rare. Reports have included a hepatocellular adenoma, a hepatic cavernous hemangioma, and a papilloma of the gallbladder (Manning, 1976).

Adenocarcinomas of the gallbladder developed in 17 of 68 guinea pigs of strains 2 and 13 over 20 months of age, but none developed in 9 noninbred guinea pigs. Significantly more females than males developed tumors (Hoch-Ligeti et al., 1979). Guinea pigs are also sensitive to liver carcinogenesis by environmental nitrosamines (Cardy and Lijinsky, 1980).

Nonneoplastic Lesions, Induced

The biliary system of guinea pigs is exquisitely susceptible to hyperplastic or neoplastic stimuli. Benign and malignant neoplasms of the biliary tract were readily induced in guinea pigs by placement of foreign bodies (choleliths, pebbles, string sutures) in the gallbladder and by administration of chemical irritants (Pityrol, lanolin, pitch pellets) (Manning, 1976). Whole-body exposure to γ- or x-ray irradiation increased the number of both adenocarcinomas and metastases in male inbred guinea pigs but not in females (Hoch-Ligeti et al., 1979).

Hepatocellular carcinomas were produced by oral administration of diethylnitrosamine in 14 of 15 guinea pigs (Argus and Hoch-Ligeti, 1963). Hepatocellular tumors were also induced by *N*-nitroso-*N*-methylurea (Yoshida et al., 1977).

Urinary System

Kidney

Nonneoplastic Lesions, Spontaneous

Chronic interstitial nephritis, also termed segmental nephrosclerosis, is a commonly observed lesion of aged guinea pigs and ascribed to be the cause of wasting disease in old pet guinea pigs (Wagner, 1976). In this disease, the animal seems to waste away. It drinks a lot of water, loses weight, hunches up, loses condition, and gradually becomes weaker until death. Its mouth often has a strong odor (Ruf, 1984). The lesions of nephrosclerosis were accelerated in guinea pigs fed an unusually high-protein diet. Clinical findings in animals with advanced kidney lesions include high blood urea nitrogen (BUN) and serum creatinine values, nonregenerative anemia, and low urine specific gravity (Percy and Barthold, 2001). Gross findings are characterized by irregularly pitted renal cortices. Possible causes include autoimmune diseases, infectious agents, and vascular diseases (Hanes, 2003). Nephrosclerosis with increased blood pressure, resembling that in hypertensive humans, has been described in Abyssinian and Hartley guinea pigs (Takeda and Grollman, 1970).

Kidneys heavily infected by the coccidium *Klossiella cobayae* may have an irregular surface with gray mottling, but in most cases, gross lesions are inapparent. Finding sporocysts in the kidney tubules is diagnostic. Subacute nephritis may accompany the protozoan infection (Vetterling, 1976). Pyelonephritis has been associated with *Corynebacterium* sp. infections, including *C. pyogenes*, *C. kutscheri*, and *C. pseudotuberculosis.*

Osseous Metaplasia Is Occasionally Observed in Kidney (Hanes, 2003)

Nonneoplastic Lesions, Induced. Studies with lead acetate have shown kidney lesions with aminoaciduria that are typical of lead poisoning (Bielecka, 1972). Autoimmune tubulointerstitial nephritis can be induced by rabbit tubular basement membrane in both strains 2 and 13, but the nephropathy develops more quickly in strain 13 (Migrom et al., 1979). National Institutes of Health (NIH) Hartley strain guinea pigs are also susceptible to experimental autoimmune tubulointerstitial nephritis (Hyman et al., 1976; Steblay, 1979).

Neoplastic Lesions, Spontaneous. Only four renal tumors have been reported: two adenocarcinomas (Mosinger, 1961), a round cell sarcoma (lymphosarcoma) (Ball and Pagnon, 1935), and an osteogenic sarcoma (Twort and Twort, 1932).

Urinary Bladder and Urethra

Nonneoplastic Lesions, Spontaneous. The most common urinary problem in guinea pigs is cystitis, evidenced by pus and blood passed when the animal's bladder is pressed (Roach, 1983). Older females are particularly affected probably because of the proximity of the urethral orifice to the anus and the likelihood of infection from fecal bacteria such as *E. coli* (Percy and Barthold, 2001). Cystitis in males is sometimes secondary to occlusion of the penile urethra by coagulum from the vesicular glands (Wagner, 1979). Obstruction of the urethra with proteinaceous concretions, probably of seminal vesicular origin, is a contributing cause of death in aged males (Wagner, 1976). Urinary calculi occur primarily in females and vary from sand-like to large concentric bladder stones. Age, sex, and immunosuppression are related to the development of urinary calculi (Peng et al., 1990; Hanes, 2003).

Neoplastic Lesions, Spontaneous. Prior to 1976, five urinary bladder tumors, four of which were malignant, had been reported. These included two transitional cell carcinomas, an epidermoid papilloma, an epidermoid carcinoma, and an anaplastic tumor (Heston and Deringer, 1952; Manning, 1976).

In 1980, an additional seven tumors were described in untreated control animals; two were heman-giopericytomas and the others were not specifically identified (Hoch-Ligeti et al., 1980).

Neoplastic Lesions, Induced. Evans (1968) reported an unspecified number of guinea pigs fed bracken fern developed transitional cell, glandular and squamous cell tumors. A single animal developed an unspecified urinary bladder tumor after 26 guinea pigs received a single injection of 7,12-dimethylbenz(*a*) anthracene (Toth, 1970). Hemangiopericytomas, transitional cell carcinomas, squamous cell carcinomas, and adenosquamous carcinomas developed in untreated controls and guinea pigs exposed to γ- and x-ray irradiation. Irradiation was associated with significantly earlier tumors, but the increase in numbers of tumor-bearing animals was not statistically significant (Hoch-Ligeti et al., 1980).

Reproductive System and Fetal Tissues

General Considerations

Infertility and fetal or newborn deaths can result from many causes including age, stress, the type of flooring, estrogen in the feed, bedding adherent to genitals, increased room temperature, nutritional deficiencies, metritis, preputial dermatitis, segmental aplasia of the uterus, or cystic ovaries. Perinatal deaths, abortion, and stillborn young are usually associated with dystocia; bacterial infections such as *Bordetella*, *Salmonella*, or *Streptococcus*; cytomegalovirus infections; birth asphyxiation; and pregnancy toxemia (Hanes, 2003).

Ovary

Nonneoplastic Lesions, Spontaneous

Ovarian and paraovarian cysts are very common in older breeding females. Cystic rete ovarii and serous cysts were found at necropsy in 54 of 71 (76%) female guinea pigs between 18 and 60 months of age (Quattropani, 1977, 1978, 1981; Shi et al., 2002). Fertility was markedly reduced in affected females over the age of 15 months. Cystic endometrial hyperplasia, mucometra, endometritis, inappropriate placental tissue, fibroleiomyomas, and alopecia frequently accompanied the cystic ovaries (Keller et al., 1987; Percy and Barthold, 2001). Ultrasonography provides a new method for the diagnosis of ovarian cysts and abdominal abscesses (Beregi et al., 1999, 2000a,b).

Nonneoplastic embryonal malformations within the ovary, also termed embryonic placentomas, were described by Loeb (1932). These structures could be misinterpreted as neoplasms. They represent parthenogenetic development of ova within the ovary, resulting in the formation of placental and embryonal structures, and are thought to occur frequently in guinea pigs. They were seen in females less than 6 months of age and most were in guinea pigs less than 4 months of age (Loeb, 1932). These malformations may become fibrotic in mature ovaries (Hanes, 2003). Estrogens appear to enhance their development (Mosinger, 1961).

Neoplastic Lesions, Spontaneous

True tumors of the ovary are rare and most (80%) those reported are teratomas (Willis, 1962; Frisk et al., 1978). Nineteen of these tumors had been reported as of 1970 (Vink, 1970). The low incidence is indicated by finding only 3 in 4,200 necropsied animals of the R9 strain (Blumenthal and Rogers, 1965) and 10 in 13,000 random-bred animals (Vink, 1970). A malignant ovarian teratoma was described by Gupta and Sarmah (1985). Five cystadenomas and a granulosa cell tumor have been reported (Jain et al., 1970; Manning, 1976; Squire et al., 1978; Hong, 1980). Tumors of the ovaries should not be confused with cystic rete tubules seen commonly in old guinea pigs (Percy and Barthold, 2001).

Oviduct, Uterus, and Vagina

The adult female has an intact, epithelial vaginal closure membrane except for the few days of estrus and at parturition. Both these events are signaled by the perforation of this membrane.

Trophoblastic giant cells derived from the fetal placenta can migrate into the myometrium and adjacent blood vessels (Hanes, 2003).

Nonneoplastic Lesions, Spontaneous

Guinea pigs appear to be predisposed to high perinatal mortality (Eveleigh et al., 1987). These deaths are often associated with dystocia and subclinical ketosis (pregnancy toxemia).

The circulatory form of pregnancy toxemia in guinea pigs is characterized by uteroplacental ischemia due to compression of the aorta caudal to the renal blood vessels by a large gravid uterus containing a near term fetus. This results in reduced blood pressure in the uterine vessels and tissue anoxia, with subsequent placental necrosis, uterine hemorrhage, thrombocytopenia, ketosis, and death. The tissue anoxia and necrosis lead to toxemia. Pregnancy toxemia is manifested by depression, acidosis, ketosis, proteinuria, ketonuria, and a lowered urinary pH from 9 to 5–6 (Percy and Barthold, 2001).

Dystocias are common problems in guinea pigs and usually occur either in young females because of the relatively large fetus or in older females because of delayed breeding (Wallach and Boever, 1983). If the first breeding of female guinea pigs is delayed past 7 or 8 months, the pubic symphysis separates with increased difficulty at parturition and fat pads occlude the pelvic canal, which lead to dystocia and death (Harkness and Wagner, 1983). Also, dystocias appear to be common sequelae of subclinical ketosis (pregnancy toxemia). Vaginal prolapse is occasionally associated with parturition (Wagner, 1976).

Metritis, or pyometra, and pyosalpinx can be caused by a variety of organisms including *Staphylococcus* spp., *Streptococcus* spp., *C. pyogenes*, *C. kutscheri*, *C. pseudotuberculosis*, and *E. coli*. The clinical signs are a mucopurulent to sanguinopurulent discharge from the vulva and a distended abdomen (Wallach and Boever, 1983).

The first deciduoma in a laboratory animal was reported in the guinea pig (Loeb, 1908). These lesions consist of proliferating decidual tissue of the uterus and were originally regarded as tumors.

Simple cysts are common in the female reproductive tract of guinea pigs. These probably represent remnants of the Wolffian duct system (Squire et al., 1978). Cyst endometrial hyperplasia, mucometra, endometritis, inappropriate placental tissue, and fibroleiomyomas were seen in 21 of 54 (39%) guinea pigs with cystic ovaries, but in only 1 of 17 (6%) guinea pigs without cystic ovaries (Keller et al., 1987). Cystic endometrial hyperplasia has been described by Ong (1987).

Nonneoplastic Lesions, Induced

Clinical and pathological features of the acute and recurrent genital herpes disease, which are similar to those seen in human genital disease, can be produced in females by inoculation with low dosages of herpes simplex virus type 2. This includes complete healing of genital lesions, histological changes in the genital epithelium and nerve tissues, latent viral infection in the dorsal root ganglia, and transmission of the virus from mother to newborn through an infected birth canal. The cervical dysplasia seen in guinea pigs is similar to that seen in humans, but none of the guinea pigs has been kept long enough for cancer to develop (Lucia and Hsiung, 1981).

Neoplastic Lesions, Spontaneous

One oviduct tumor, an adenoma, has been reported (Manning, 1976). Uterine tumors are usually benign and have mesenchymal origin. Although a variety of tumors have been observed in

the uterus, most are fibromas or leiomyomas. Malignant uterine tumors are rare and often consist of poorly differentiated mesenchymal cells, with extension into the peritoneal cavity (Percy and Barthold, 2001). Seven tumors were described in the uterus of R9 strain animals by Rogers and Blumenthal (1960). These included two leiomyomas, an adenomyoma, a leiomyosarcoma, a fibrosarcoma, a myxosarcoma, and a mixed mesenchymal tumor. Other tumors reported include a sarcoma, a fibromyoma, a myxofibroma, a fibroma, and two adenocarcinomas (Manning, 1976). Cervical lymphoblastic lymphoma has been seen in aged animals (Wolff et al., 1988).

Neoplastic Lesions, Induced

Prolonged estrogen administration has induced benign tumors in the uterus of guinea pigs, including fibromas, fibromyomas, and leiomyomas (Manning, 1976; Field et al., 1994).

Prepuce

Nonneoplastic Lesions, Spontaneous

Wood shavings or sawdust used for bedding can become lodged in the prepuce or around the anus in the male guinea pig and form a hard mass that prevents erection (Harkness and Wagner, 1983). Male guinea pigs occasionally develop infections due to these foreign materials in the preputial folds. Long-standing infections may cause adhesions and infertility (Wagner, 1976).

Vesicular Glands

Nonneoplastic Lesions, Spontaneous

The vesicular glands of the adult male are bilateral, smooth, and transparent and extend approximately 10 cm into the abdominal cavity from their origin in the accessory sex glands. They can be confused with the uterus by an inexperienced observer (Harkness and Wagner, 1983). Their large size and the normal presence of open inguinal canals contribute to their herniation into the scrotum.

Testes

Nonneoplastic Lesions, Induced. Strain 13 guinea pigs are susceptible to the development of experimental autoimmune orchitis by immunization with isogenic and allogenic spermatozoa and with sperm autoantigens (Carlo et al., 1976; Toullet and Voisin, 1979). Isoimmunization with testicular material in FCA resulted in subsequent chronic orchitis (Parsonson et al., 1971). Testicular atrophy is seen with analgesics (Boyd, 1970).

Neoplastic Lesions, Spontaneous. Testicular tumors of any type are extremely rare in the guinea pig (Squire et al., 1978). There has been a single report of an embryonal carcinoma of the testis (Blumenthal and Rogers, 1965).

Musculoskeletal System

Bone and Joints

General Considerations—When guinea pigs are reluctant to move, malnutrition, scurvy, vitamin E deficiency, osteoarthritis, spinal trauma, fractures, myopathy, muscular dystrophy, or bacterial infections such as *Bordetella* and *Salmonella* may be the cause (Hanes, 2003). Osteoarthritis and its development were studied early in the guinea pig (Silverstein and Sokoloff, 1958).

Nonneoplastic Lesions, Spontaneous

Broken limbs often occur when guinea pigs not raised in wire-bottomed cages are placed in such cages (Harkness and Wagner, 1983) or are incorrectly handled (Roach, 1983). Vertebral sub-luxation has been reported (Hammons, 1979). Bone lesions of scurvy (vitamin C deficiency) include hemorrhage of the epiphyseal plate accompanied by subcutaneous and periarticular hemorrhages (Gleiser, 1974). Spontaneous cartilage degeneration, osteoarthrosis or osteoarthritis of the femoro-tibial joint resembles that of osteoarthritis in humans and provides a useful model for this disease (Gupta et al., 1972; Bendele and Hulman, 1988; Bendele et al., 1989, 1999). Septic arthritis has been associated with *Streptococcus* spp.; *Corynebacterium* spp., including *C. pyogenes*, *C. kutscheri*, and *C. pseudotuberculosis*; and *S. moniliformis.*

Metastatic calcification is often seen as an incidental finding especially in the hind legs (Hanes, 2003). Characterized by calcium deposits in joints, muscles, and various soft tissues including those of internal organs, such as the heart, aorta, lungs, kidneys, stomach, and colon, it occurs most often in adult males over the age of 1 year. The guinea pig loses weight, has stiff joints, and usually dies. The causes are considered imbalances of magnesium, potassium, calcium, and phosphorus, aggra-vated by too much vitamin D (Ruf, 1984). It is believed that hyperphosphatemia results from the inability of the guinea pig to conserve fixed bases by excreting ammonia in the urine; thus, the low-base reserve impairs normal urinary excretion of phosphorus (Fraser et al., 1986).

Nonneoplastic Lesions, Induced

Progressive chronic osteoarthritis has been induced experimentally in the femorotibial joints of guinea pigs by partial medial meniscectomy (Bendele, 1987; Bendele and White, 1987; Bendele et al., 1999).

Neoplastic Lesions, Spontaneous

Spontaneous tumors of bones are also rare (Olcott and Papanicolaou, 1943; Olson and Anver, 1980; Hong and Liu, 1981). Manning (1976) cited five tumors that included an enchondroma, an osteogenic sarcoma (osteosarcoma), a chondrosarcoma, an osteochondrosarcoma, and a neurogenic fibrosarcoma. An osteosarcoma was described by Jolivet (1988). Multiple extraskeletal osteogenic sarcomas were reported in a 2-year-old female (Cook et al., 1982) and metabolic calcifications in a closed colony (Sparschu and Christie, 1968).

Neoplastic Lesions, Induced

A few osteosarcomas and chondrosarcomas were produced at injection sites by methylcholan-threne in the subcutaneous and muscular tissues (Blumenthal and Rogers, 1965).

Skeletal Muscle

Nonneoplastic Lesions, Spontaneous

Muscular dystrophy is characterized by lameness, stiffness, and refusal to move due to vitamin E deficiency (Ruf, 1984). In a colony of 150 animals, 54 were affected with a myopathy resem-bling nutritional muscular dystrophy. Fifty percent of those affected died. Major clinical signs were depression, conjunctivitis, and reluctance to move. Lesions were widespread throughout skeletal

and cardiac musculature. There was pallor or pale longitudinal streaks in otherwise normal muscle. Microscopically, the lesions were characterized by widespread degeneration and coagulation necrosis with myositis and regeneration in various muscle groups (Ward et al., 1977).

Many cases of myopathy have been observed and reported in guinea pigs in a number of different experimental regimens that have no apparent common etiology. The large muscles of the rear legs are most involved. The lesions are characterized by general muscle degeneration having a varied degree of swelling, loss of cross striations, fragmentation of fibers, and vacuolation (Webb, 1970; Wagner, 1976).

Myositis was characterized clinically by swelling and pain in the large muscle groups of the hind legs, followed by front leg involvement, prostration, and death in newly purchased animals. Gross ecchymotic hemorrhages and edema were observed in affected muscles. Microscopically, hemorrhage and edema of the skeletal muscles were the prominent changes with infiltration by acute- to subacute-type inflammatory cells. A virus was suspected but could not be isolated (Saunders, 1958). Guinea pig pox-like virus was isolated from young guinea pigs that spontaneously developed a fibrovascular proliferation in the thigh muscles (Van Hoosier and Robinette, 1976).

Spontaneous arthrogryposis has been reported in a guinea pig. The disease was tetramelic and apparently due to the reduced size of skeletal muscles and their individual fibers. There was an associated myelodysplasia characterized by focal duplication of the spinal canal and no central canal in other areas of the cord (Doige and Olfert, 1974).

Neoplastic Lesions, Spontaneous

Primary tumors of the skeletal muscle are very rare. Kroning and Wepler (1938) reported a lipomyxofibroma invading the psoas muscle in a 3-year-old male guinea pig.

Nervous System and Special Sense Organs

Brain, Spinal Cord, and Nerves

Nonneoplastic Lesions, Spontaneous

Lymphocytic choriomeningitis is a naturally occurring arenaviral disease of the nervous system. The clinical signs include ruffled fur, huddled posture, palpebral edema, conjunctivitis, tremors, convulsions, and hind leg paralysis. Gross findings may be minimal or consist of interstitial pneumonia, pulmonary edema, pleural exudate, fatty liver, and enlarged spleen. Microscopically, marked lymphocytic infiltrations are observed in the meninges, especially at the base of the brain and in the choroid plexus, as well as the liver, adrenals, kidneys, and lungs (Van Hoosier and Robinette, 1976).

A poliovirus-like disease, called guinea pig lameness, was associated with a flaccid paralysis and loss of weight. Microscopic changes consisted of a meningomyeloencephalitis affecting the lumbar spinal cord and medulla oblongata. A median and ulnar neuropathy has been reported (Fullerton and Gilliatt, 1967).

Alpha-mannosidosis is a lysosomal storage disorder resulting from the deficient activity of lysosomal alpha-mannosidase. The disease is characterized by neuronal storage leading to progressive mental deterioration. The affected guinea pigs closely resemble the human disease and will provide a convenient model for investigation of new therapeutic strategies for neuronal storage diseases, such as enzyme replacement and gene replacement therapies (Crawley et al., 1999).

Cerebral larval migrans with multifocal malacia and eosinophilic granulomatous inflammation were observed in a colony due to *B. procyonis* eggs in wood shavings bedding contaminated by raccoon feces. The affected guinea pigs manifested cachexia, stupor, hyperexcitability, lateral recumbency, and opisthotonus (Van Andel et al., 1995).

Spontaneous encephalomyelitis caused by *Encephalitozoon* (*Nosema*) *cuniculi*, a microsporidian organism (Moffatt and Schiefer, 1973), and encephalitis caused by *T. gondii*, the tissue form of the feline coccidia and by Hendra virus, have been observed in guinea pigs (Vetterling, 1976; Williamson et al., 2001, respectively). Meningoencephalitis is sometimes associated with *S.* (*Diplococcus*) *pneumoniae*.

Nonneoplastic Lesions, Induced

NIH Hartley strain guinea pigs were susceptible to experimental allergic encephalomyelitis (Lisak et al., 1975). Chronic demyelinating optic neuritis can be produced in juvenile strain 13 guinea pigs by intradermal injections of the spinal cord emulsified with Freund's adjuvant. After several months, the optic neuritis is followed by encephalomyelitis. Injections of adult strain 13 animals resulted in acute optic neuritis and allergic encephalomyelitis with death within 4–6 weeks (Rao et al., 1979).

Segmental demyelination and axonal degeneration of the peripheral and cranial nerves in guinea pigs have been produced by lead acetate (Fullerton, 1966; Gozdzik-Zolnierkiewicz and Moszynski, 1969). Asphyxia neonatorum in fetal animals resulted in signs of neural damage and a variety of histopathological changes (Windle and Becker, 1943).

Neoplastic Lesions, Spontaneous

Tumors of the nervous system are apparently very rare (Squire et al., 1978). Only one tumor was reported in the central nervous system, a teratoma of the pons (Lutz, 1910). Interestingly, no gliomas, which are the most frequent tumors in most other species, were observed (Squire et al., 1978). Three tumors of the nerve sheaths were described. All three appeared to be neurilemmomas, although one of the inner abdominal walls had a diagnosis of glioma. The other two occurred in the mesentery (Manning, 1976).

Ear

Nonneoplastic Lesions, Spontaneous

Suppurative ear infections (otitis media) are seen occasionally with torticollis, incoordination, and circling behavior if the infection extends to the inner ear. A number of bacterial agents can be involved. Among the most common are those that are also capable of causing respiratory disease such as *S. zooepidemicus*, *S. pneumoniae*, and *B. bronchiseptica* (Wagner et al., 1976; Wagner, 1979; Roach, 1983; Boot and Walvoort, 1986).

The waltzer strain of guinea pig is characterized by a tendency to "waltz" or whirl and loss of hearing. Rotation does not elicit a nystagmus response and tilting does not evoke counterrolling of the eyes. There is a loss of the Preyer reflex by 4–6 weeks. They have an impaired swimming ability and righting reflexes. The hearing loss, which affects the high-frequency range first, is complete by 42 days. Vestibular capacity is decreased at birth and progresses to complete loss of function. The hair cells of the organ of Corti degenerated, followed by supporting cell degeneration and depopulation of neurons in the spiral ganglion (Ernston, 1970, 1971a,b, 1972a,b,c).

Nonneoplastic Lesions, Induced

The guinea pig ear and associated structures provide an important animal model for ototoxicity and acoustic trauma studies. The petrous bone (otic capsule) is easily entered and dissected away. The guinea pig has been used to elucidate the pathophysiology of drugs that cause deafness and

vestibular disorders, including the aminoglycosidic antibiotics such as neomycin, gentamicin, and other members of the streptomycin family, the diuretic ethacrynic acid, quinine, and salicylates (McCormick and Nuttall, 1976). A variety of protocols continue to be used to study mechanisms of ototoxicity especially by compounds such as the anticancer drug, cisplatin, and other potential therapeutics (Güneri et al., 2003; Sergi et al., 2003; Ekborn et al., 2004).

Eye

Nonneoplastic Lesions, Spontaneous

The most common eye disorders in guinea pigs are conjunctivitis, corneal ulcers, and keratitis (Kohn, 1974; Roach, 1983; Cullen et al., 2000). A wide variety of microorganisms have been isolated from eye lesions, including coliforms, *S. zooepidemicus*, hemolytic *Streptococcus* spp., *Micrococcus* spp., *Staphylococcus aureus*, *P. multocida*, *Pasteurella haemolytica*, *S. typhimurium*, *S. enteritidis*, *S. dublin*, *M. pulmonis*, and *Proteus* spp. (Wagner, 1976). Guinea pig inclusion conjunctivitis is caused by *Chlamydophila caviae*, formerly termed *Chlamydia psittaci* (Eidson, 2002) and usually is a mild, self-limiting ocular disease in animals 4–8 weeks old (Deeb et al., 1989).

Congestion of the lower conjunctival sac following irritation results in a protrusion termed as red or pea eye. Protrusion of the lower conjunctival sac can also be observed in some guinea pigs as a result of excess retrobulbar fat (Richardson, 2000).

Cataracts are occasionally seen in young and adult animals as incidental findings, irrespective of the diet (Wagner, 1976). An autosomal dominant trait possibly associated with diabetes or L-tryptophan deficiency has been identified (Hanes, 2003). Senile cataracts develop in old pet guinea pigs at ages 6–8 years (Ruf, 1984). Opacities of the cornea can result from drying of the eye during long-term anesthesia (Hanes, 2003).

Bilateral osseous choristomas of the ciliary body were observed in 3 guinea pigs of a research colony of about 200 animals. One of these animals had also bilateral cataracts (Griffith et al., 1988).

Osseous metaplasia of the eye has been observed (Hanes, 2003). Formation of heterotrophic bone was reported in the ciliary body of an aged guinea pig (Brooks et al., 1990).

Nonneoplastic Lesions, Induced

Optic disk swelling, a protrusion of the optic nerve head into the vitreous with varying degrees of vascular and peripapillary retinal changes can be produced in guinea pigs with β,β-iminodipropionitrile (IDPN) (Parhad et al., 1986). Optic disk swelling is seen also in association with optic neuritis and papilledema induced by the intradermal injection of the spinal cord emulsified with Freund's adjuvant (Rao et al., 1979). NIH Hartley strain guinea pigs were susceptible to experimental autoimmune uveitis (McMaster et al., 1976).

The guinea pig model of allergic conjunctivitis is used to study the effectiveness of therapeutic agents (Schoch, 2003).

Neoplastic Lesions, Spontaneous

A lymphosarcoma of the choroid and two intraocular reticulosarcomas were reported in guinea pigs by Congdon and Lorenz (1954). Widespread systemic involvement in malignant lymphoma can accompany or precede recognizable intraocular lesions. A corneal dermoid was observed in a hairless guinea pig (Otto et al., 1991). Scleral dermoids have been observed in four animals (Gupta, 1972).

Endocrine System

Pancreas

Nonneoplastic Lesions, Spontaneous

Acute spontaneous diabetes mellitus was characterized by polydipsia, polyuria, hyperglycemia, glycosuria, elevated glucose tolerance tests, and elevated plasma triglycerides in Abyssinian guinea pigs. It resembled human juvenile diabetes mellitus and spread from animal to animal possibly caused by an unidentified virus (Munger and Lang, 1973; Lang and Munger, 1976; Lang et al., 1977). Diabetes represents a possible cause of infertility (Percy and Barthold, 2001).

Fatty deposits in the pancreas increase markedly with age. This increase is associated with a proportional increase in islet tissue and a decrease in exocrine pancreatic tissue (Wagner, 1976).

Neoplastic Lesions, Spontaneous

Very few endocrine tumors have been reported. The most frequent were islet cell adenomas. These benign pancreatic islet cell tumors were found in 0.7% of animals of the N: Hart NIH Hartley strain guinea pigs and in 3.5% of strain 13 animals that survived longer than 2 years (Yoshida et al., 1979).

Neoplastic Lesions, Induced

Pancreatic adenocarcinomas have been induced by *N*-methyl-*N*-nitrosourea (Reddy et al., 1974; Reddy and Rao, 1975).

Adrenal

Guinea pigs have normally large prominent adrenal glands, which must be considered when evaluating this organ at necropsy:

Nonneoplastic Lesions, Spontaneous. Occasional cortical adenomas or hyperplastic nodules are seen in older guinea pigs (Squire et al., 1978). A number of these lesions were reported in male guinea pigs some years after castration. Manning (1976) considered these lesions most likely to be castration-induced hyperplastic nodules. Enlarged adrenal glands with hemorrhage and cortical necrosis can result from ketosis.

Neoplastic Lesions, Spontaneous. Occasional cortical adenomas or hyperplastic nodules are seen in older guinea pigs (Squire et al., 1978). A number of these lesions were reported in male guinea pigs some years after castration. Manning (1976) considered these lesions to most likely be castration-induced hyperplastic nodules. Other reports included three cortical adenomas, a cortical carcinoma, and an unclassified tumor (Manning, 1976).

Thyroid and Parathyroid

Nonneoplastic Lesions, Induced. NIH Hartley strain guinea pigs (McMaster and Lerner, 1967; McMaster et al., 1967) and strain 2 (Braley-Mullen et al., 1975) guinea pigs are highly susceptible to experimental autoimmune thyroiditis.

Neoplastic Lesions, Spontaneous. Spontaneous thyroid tumors are also rare. One papillary adenoma was observed in a Hartley strain animal (LaRegina and Wightman, 1979). One carcinoma, possibly of parafollicular origin, was described by Zarrin (1974). No parathyroid tumors were reported (Manning, 1976).

Pituitary

Neoplastic Lesions, Spontaneous. Spontaneous pituitary tumors are very rare; if they occur. None have been reported (Manning, 1976; Squire et al., 1978).

Integumentary System and Soft Tissues

Skin

Nonneoplastic Lesions, Spontaneous

Guinea pigs born in wire-bottom cages learn to walk without mishap, but naive animals placed in such cages often fall through the mesh and break or lacerate their limbs. The latter animals may also exhibit hair loss, footpad ulcers, decreased reproduction, and effects from stress (Harkness and Wagner, 1983).

The most common foot problem is peeling of the skin and thickening of the hock with or without ulceration. It seems to be associated with unsuitable bedding and the presence of moisture. Hard damp straw will cause the problem (Roach, 1983). Marked hyperkeratosis of the footpads is occasionally observed. In some cases, the horny growths approached 2 cm in length. All cases were aged animals confined to laboratory cages (Wagner, 1976).

Abscesses are most commonly found around the jaw, neck, and feet. Abscesses near the throat are of two types. One type is the result of streptococcal infections, and usually involves the cervical lymph nodes. The other type results from inflammation around food materials caught in little pockets or indentations in the cheeks (Ruf, 1984). Abscesses of the skin and feet are often associated with *S. aureus* infections (Taylor et al., 1971). A chronic ulcerative pododermatitis can result in which the foot lesions persist indefinitely, periodically ulcerating and bleeding and producing abscesses in the draining lymph nodes, osteoarthritis, and generalized amyloidosis (Gleiser, 1974). Wounds and abscesses on the back may be the results of fighting between boars with a group of overcrowding (Roach, 1983).

Guinea pigs are close to humans in responsiveness to irritative dermatitis agents (Nixon et al., 1975). Dermatitis occurs as a result of a variety of conditions in guinea pigs and can be caused by vitamin C deficiency, bacterial infections, external parasites, or ringworm. External parasites include fleas, ticks, lice (*Gliricola porcelli*, *Gyropus ovalis*, or *Tremenopon hispidium* [*jenningsi*]), and mites (*Trixacarus caviae*, *Chirodiscoides caviae*, or *Demodex caviae*) (Fuentealba and Hanna, 1996). Heavily louse-infested animals may have a rough, dry hair coat and may scratch excessively. The signs of ringworm include patchy loss of hair and scaling of the hair or dark spots around the eyes and other parts of the body. Usually, there is mild to severe itching, often leading to secondary scabbing and bleeding. The most common causes of ringworm are *Trichophyton mentagrophytes* and *Microsporum gypseum* (Lee et al., 1978; Wagner, 1979; Roach, 1983; Ruf, 1984; Fraser et al., 1986; Pollock, 2003). A case of dermal cryptococcosis caused by *Cryptococcus neoformans* involved the nose (Van Herck et al., 1988).

Alopecia is a manifestation of several conditions. Alopecia, usually bilateral, develops to a degree in all guinea pigs in late pregnancy and is considered a normal phenomenon in aged breeding females. The condition appears to be due to reduced anabolism of maternal skin associated with fetal growth. The hair loss frequently occurs over the back and rump. Also alopecia can accompany cystic rete ovarii (Percy and Barthold, 2001). Adequate zinc levels in the diet are required for hair growth (Wallach and Boever, 1983). Protein deficiency and other nutritional or genetic factors may be involved (Hanes, 2003). Thinning of the hair around the time of weaning is common in young guinea pigs during the period of transition as the baby fur is lost and the coarse guard hairs of adult fur appear (Wagner, 1976, 1979).

Loss of hair from pulling, stripping, barbering, or hair chewing occurs in some colonies, especially in animals reared in wire cages. It is thought to be mainly due to boredom. It can be self-inflicted or mutually inflicted (Roach, 1983).

Ear chewing occurs as vice associated with excessive aggressiveness of one or more individuals in a cage or primary enclosure (Wagner, 1976). Severe trauma and amputation of the pinnae can result (Percy and Barthold, 2001). This practice can lead to skin infections as well as to serious interference in the identification of individual animals from loss of ear notches or ear tags.

Dense populations of sebaceous glands, marking glands, are located circumanally and on the rump. Activity of these glands can result in excessive accumulation of sebaceous secretions in the folds of the circumanal and genital region of adult male guinea pigs. These folds must be cleaned periodically in older males to preclude infections and unpleasant odors.

Nonneoplastic Lesions, Induced

Guinea pigs are used as models for studies of phototoxicity, thermal burns, chemical burns, and wound healing. For example, the guinea pig provides a model for experimental hypertrophic scars (Aksoy, 2002). These studies are useful in developing products for the treatment of ulcers, burns, wounds, and scars (Wormser et al., 2000a,b; Lindblad, 2001; Tan et al., 2002). A hairless strain of guinea pig with normal thymic activity provides a useful dermal research subject. Their hair follicles produce rudimentary hairs. Their skin is nonpigmented and fragile (Hanes, 2003). Athymic or hypothymic hairless guinea pigs also have been observed. Animals with the thymic mutations were agammaglobulinemic and died of infections usually associated with immuno-deficiencies (Reed and O'Donoghue, 1979). Injections of a mixture of droperidol and fentanyl citrate (Innovar-Vet) have resulted in self-mutilation, distal necrosis at the injection site, and amputation of the leg (Leach et al., 1973; Thayer et al., 1974; Newton et al., 1975; Wagner, 1979). Infections by *T. mentagrophytes* provide useful models for mycotic dermatitis (Treiber et al., 2001; Pollock, 2003).

Neoplastic Lesions, Spontaneous

Prior to 1965, spontaneous skin tumors were considered rare in guinea pigs (Blumenthal and Rogers, 1965); however, examinations of Hartley strain animals at Fort Detrick, Maryland, have revealed 29 trichofolliculomas (Manning, 1976). Twenty-one trichofolliculomas were noted at necropsy of 7670 guinea pigs (Ediger et al., 1971). Other skin and subcutaneous tumors reported include cutaneous papillomas, penile papillomas, trichoepitheliomas, sebaceous adenomas, an undifferentiated adenocarcinoma, an undifferentiated carcinoma, a fibroma, a fibrolipoma, lipomas, fibrosarcomas, a schwannoma, and an undifferentiated sarcoma (Manning, 1976; Zwart et al., 1981; Percy and Barthold, 2001). A complex adnexal tumor with both sebaceous and apocrine differentiation has been reported (Allison and Moeller, 1993).

Neoplastic Lesions, Induced

Epidermal neoplasms have proven very difficult to induce by topical application. The few that have been reported required a minimum of 2 years of application and large doses of carcinogen (Stenbeck, 1970).

Mammary Gland

Nonneoplastic Lesions, Spontaneous. Bacterial mastitis is a fairly common disease of guinea pigs, particularly in sows during early lactation. It is characterized by red to purple enlarged, firm, congested, edematous mammary glands and caused by infections from a variety of organisms, including *Staphylococcus* spp., *Streptococcus* spp., and *Corynebacterium* spp. (Kinkler et al., 1976; Wagner, 1979).

Neoplastic Lesions, Spontaneous. Tumors of the mammary gland are relatively common and occur in both males and females (Hoch-Ligeti et al., 1986; Percy and Barthold, 2001). Blumenthal and Rogers (1965) reported that 12 of the 140 tumors reported in guinea pigs were mammary gland lesions. Tumors that have been described are adenomas, a cystadenoma, fibroadenomas, an adeno-fibroma, fibrocytadenomas, papillary adenomas, papillary cystadenomas, adenocarcinomas, a liposarcoma, a carcinosarcoma, and a malignant mixed tumor. The most common mammary tumors are adenocarcinomas, several of which have been reported in males. Nearly all the carcinomas were believed to be of ductal origin (Andrews, 1976; Manning, 1976; Squire et al., 1978). Although many are of low-grade malignancy and remain localized, metastases have been observed in the adjacent regional lymph nodes (Hoch-Ligeti et al., 1986; Percy and Barthold, 2001).

Subcutis

Nonneoplastic Lesions, Spontaneous

Subcutaneous abscesses are common lesions in guinea pigs from a variety of injuries. The microorganisms involved vary considerably, but may include *Staphylococcus* spp., *Streptococcus* spp., *Corynebacterium* spp., *Sphaerophorus necrophorus*, and *E. coli* (Wallach and Boever, 1983).

Soft tissue calcification is a disease of nutritional origin characterized by metastatic calcification in various soft tissues, including the muscles, myocardium, joints, stomach, aorta, lungs, kidneys, uterus, sclera, and, most striking, at the colonic flexure. In some animals, the soft tissue mineralization may be confined to the elbows and ribs (Percy and Barthold, 2001). Microscopically, mineralization may be seen in almost any organ, most often in adult males over the age of 1 year. The condition occurs when there is an imbalance in magnesium, calcium, and phosphorus interrelationships and is corrected by providing an adequate amount of magnesium (Lane-Petter et al., 1967).

Nonneoplastic Lesions, Induced

Soft tissue calcification is a toxic effect of the Argentinian plant *Solanum malacoxylon* (Camberos et al., 1970).

Neoplastic Lesions, Induced

Subcutaneous sarcomas, including fibrosarcomas, a fibromyxoliposarcoma, and liposarcomas, were produced at the site of injection by methylcholanthrene (Shimkin and Mider, 1941).

Hematopoietic and Lymphoid System

Thymus

The thymus in immature animals is located subcutaneously in the neck on either side of the trachea, where it is easily removed by surgery (Harkness and Wagner, 1983). Degeneration of thymocytes adjacent to Hassall's corpuscles is a common finding, especially in young animals, unrelated to treatment (Percy and Barthold, 2001).

Kurloff cells, also termed Foa–Kurloff cells, are mononuclear leukocytes containing intracytoplasmic inclusions (Kurloff bodies) of a glycoprotein composition. These cells proliferate during estrogenic stimulation and are found in highest numbers in the placenta, where they may have a function protecting fetal antigens from sensitized maternal lymphocytes and immune globulins. These cells are most prominent during late pregnancy. They are commonly seen in histological sections of the bone marrow, thymus, spleen, and placenta and may originate from the thymus gland (Wagner, 1979; Harkness and Wagner, 1983). Kurloff cells may be the counterpart to natural killer

cell in other species and play a role in the low frequency of neoplasia in guinea pigs (Percy and Barthold, 2001). It is important to be aware of these special lymphoid cells and not mistake them for abnormalities such as lupus erythematous cells in blood samples.

Lymphoid Tissues, Lymph Nodes, and Spleen

Nonneoplastic Lesions, Spontaneous

Cervical lymphadenitis primarily caused by β-hemolytic group C *S. zooepidemicus* results in abscesses of the cervical lymph nodes and occasionally other nodes. The lymph nodes are enlarged and full of pus, from which the organisms can readily be isolated. Other organisms that have been isolated are *Y.* (*Pasteurella*) *pseudotuberculosis*, *S. moniliformis*, and *S. necrophorus.*

Sick guinea pigs should be palpated for enlarged mesenteric lymph nodes, which may indicate mesenteric lymphadenitis resulting from infections caused by *Y. pseudotuberculosis* or phycomycosis caused by a fungus in hay. Pseudotuberculosis can spread through the blood to the liver and spleen. Young guinea pigs with swollen mesenteric lymph nodes should be held for 30 days before being discarded, since phycomycosis usually resolves within a month of infection with no ill effects (Hime and O'Donoghue, 1979). Phycomycosis (mucormycosis) is caused by *Mucor* spp. and *Absidia* spp. and results in a characteristic lymphadenitis and granulomatous inflammation of the viscera. This mycotic infection of the mesenteric lymph nodes must be differentiated microscopically from pseudotuberculosis. Other causes of lymphadenitis in various parts of the body include *Staphylococcus* spp.; *Pasteurella* spp.; *Pseudomonas* spp.; and *Corynebacterium* spp. infections, including *C. pyogenes*, *C. kutscheri*, and *C. pseudotuberculosis*; and toxoplasmosis.

Enlargement and focal necrosis of the spleen often accompanies bacterial diseases such as salmonellosis (*S. typhimurium*, *S. enteritidis*, *S. dublin*, and *S. limete*) and pseudotuberculosis (*Y. pseudotuberculosis* and *Y. enterocolitica*).

Nonneoplastic Lesions, Induced

Cervical lymphadenitis in guinea pigs was induced experimentally with Lancefield's group C streptococci but not with group E streptococci (Olson et al., 1976).

Feeding dried plant material of *Swainsona galegifolia* produces vacuolation of circulating lymphocytes as well as vacuolar lesions in the kidneys, liver, and neurons of the central nervous system. This vacuolation is a form of the lysosomal storage disease alpha-mannosidosis caused by the indolizidine alkaloid swainsonine, a potent inhibitor of lysosomal α-ᴅ-mannosidase (Huxtable, 1969; Huxtable and Gibson, 1970; Huxtable and Dorling, 1982). Spontaneous alpha-mannosidosis occurs in guinea pigs with deficient lysosomal alpha-mannosidase activity. Affected guinea pigs have neural and visceral changes that closely resemble the human disease (Crawley et al., 1999; Muntz et al., 1999).

Germfree guinea pigs have hypoplastic lymph nodes along the gastrointestinal tract. White blood cell counts are lower than in conventional animals. The differential white cell counts are also different (Hanes, 2003).

Neoplastic Lesions, Spontaneous

Disseminated lymphomas and lymphocytic leukemias occur in middle-aged to old guinea pigs (Blumenthal and Rogers, 1967; Ediger and Rabstein, 1968; Rhim and Green, 1977; Hong et al., 1980; Steinberg, 2000). Blumenthal and Rogers (1965) reported an incidence of 7%, including the cases found in the literature. Lymphatic leukemia had an occurrence of 3.6% in the strain 2 guinea pig and an occurrence of 6.7% in strain 13 (Congdon and Lorenz, 1954). The clinical signs are anemia, anorexia, and enlargement of peripheral lymph nodes. Leukocytosis with counts of 50,000–180,000/mm^3 or

greater with a preponderance of lymphoblastic cells is typical of leukemic blood samples (Percy and Barthold, 2001). A needle aspirate can be an aid for the diagnosis of the disease in guinea pigs (McEwan and Callahan, 1993). At necropsy, grossly enlarged lymph nodes, spleen, and liver are usually present. Microscopically, lymphoblastic cells infiltrate many tissues, especially perivascularly, such as in the kidney, liver, interstitium of the lung, heart, kidney, thymus, eyes, and adrenals. The spleen, cervical and mesenteric lymph nodes, Peyer's patches, and bone marrow are all heavily infiltrated (Van Hoosier and Robinette, 1976). Leukemia occurs on rare occasions as a spontaneous disease in various inbred and noninbred strains in young adult animals (Green et al., 1973; Debout et al., 1987; Day and Briggs, 1997; Percy and Barthold, 2001).

A virus has been isolated that produces acute lymphoblastic or stem cell leukemia in strain 2 or F_1 hybrid guinea pigs (Kaplow and Nadel, 1979). Four distinct morphological types of tumors developed in animals injected with the Snijders transplantable leukemia are as follows: (1) lymphoblastic leukemia, (2) aleukemic leukemia, (3) lymphosarcoma, and (4) "lymphosarcoma resembling that of man" (Manning, 1976). Similarities of strain 2 leukemias to human leukemias include origin from bone marrow, rapidly fulminating course with lymphocyte counts of 25,000–100,000, frequent infiltration of practically all organ systems, and response to therapeutic agents (Opler, 1971; Kaplow and Nadel, 1979).

A histicytic lymphosarcoma was reported by Kitchen et al. (1975). Robinson (1976) reported two primary spleen tumors: a splenoma and a sarcoma. An additional eight primary splenic tumors were reported in untreated control animals of both sexes. They were three glomerular vascular tumors, three sinusoidal hemangioendothelioma, one hemangiosarcoma, and a chondromatous tumor (Hoch-Ligeti et al., 1981). Leukemias are well established as a result of chemical carcinogenesis, and the guinea pig provides as excellent model for human for the acute onset version (Murphy and LoBuglin, 1977).

Neoplastic Lesions, Induced

Whole-body irradiation with γ- or x-rays increased the rate of occurrence and number of splenic tumors after the age of 30 months. Lipomatous tumors occurred in addition to the types observed in untreated control animals. A significantly greater number of tumors occurred in inbred strains than in the noninbred strains (Hoch-Ligeti et al., 1981).

Nutrition and Metabolic Diseases

Nonneoplastic Lesions, Spontaneous

Vitamin C (Ascorbic Acid)

Guinea pigs, like primates, are genetically deficient in the enzyme L-gulonolactone oxidase in the glucose to vitamin C pathway. They are unable to convert L-gulonolactone to L-ascorbic acid. Also, ascorbic acid turnover is rapid and tissue storage is insufficient for periods of inadequate intake. Therefore, they require adequate dietary ascorbic acid, which is approximately 10 mg/kg body weight per day for maintenance and 30 mg/kg body weight per day during pregnancy. The ascorbic acid can be supplied in the feed, added to the water, or by fresh vegetables such as cabbage, kale, or oranges. Carrots and lettuce are not good sources of vitamin C. Ascorbic acid should be placed in water daily because of the loss of the vitamin's activity. Feed containing ascorbic acid should be properly stored and used within 90 days of milling. Guinea pigs should not be fed diets indicated for other species. Rabbit food, for example, contains no ascorbic acid, and for guinea pigs, excess levels of vitamin D (Navia and Hunt, 1976; Harkness and Wagner, 1983). Guinea pigs fed feed accidentally autoclaved that destroyed the vitamin C have developed scurvy. Vitamin C (ascorbic acid) deficiency results in increased susceptibility to infectious agents such as *S. zooepidemicus*, *S. pneumoniae*, *B. bronchiseptica*, and *K. pneumoniae* with inflammation especially of the lungs and cervical lymph nodes.

Signs of vitamin C deficiency are unsteady gait, painful locomotion, hemorrhage from gums, swelling of costochondral junctions, and emaciation. Deaths are common. Lesions include serosal, subcutaneous, and periarticular hemorrhages. Microscopically, there are hemorrhages of the epiphyseal plate, disarray of the cartilage columns, and fibrosis of the marrow in areas of active osteogenesis (Navia and Hunt, 1976; Fraser et al., 1986). Many guinea pigs die of acute bacterial infections before they can die of classic scurvy (Gleiser, 1974).

Nine episodes of subclinical scurvy were observed in 28 guinea pigs. The clinical signs were diarrhea, weight loss, and dehydration. The classic lesions of hemorrhage in the subperiosteum, skeletal muscles, joints, and intestine were not seen. Microscopically, the epiphyses were attenuated and irregular. The amount of osteoid was less than normal. Many guinea pigs had acute enteritis. All episodes were associated with either autoclaving food without adequate supplementation or other inadequate feed management practices (Clarke et al., 1980).

Vitamin D

Hypervitaminosis D is characterized by medial calcification of the major elastic arteries and soft tissue calcification.

Vitamin E

Guinea pigs are very sensitive to α-tocopherol deficiency. The clinical signs are stiffness, reluctant movement, and hind limb weakness. Affected sows may have marked reduction in reproductive performance. Testicular degeneration is observed after longer periods of deficiency. Severely affected animals may die within a week of the onset of clinical signs. Microscopically, the skeletal muscle has coagulation necrosis (Wagner, 1979; Percy and Barthold, 2001). When fed a diet with a combined deficiency of vitamins E and C, weanling guinea pigs developed a distinct clinical syndrome in which they became paralyzed and died (Hill et al., 2003).

Vitamin A

Hypervitaminosis A is characterized by a high incidence of gross structural malformations in fetuses and newborn animals (Shenefelt, 1972).

Zinc

Zinc deficiency results in alopecia, especially in female guinea pigs at about the 50th day of gestation. The hair will usually regrow in 2 or 3 weeks following parturition if the animals are on an adequate zinc diet (Wallach and Boever, 1983).

Ketosis

Ketosis is a common metabolic abnormality in obese guinea pigs after stress factors such as fasting, shipping, or changes in feeding routines. It primarily affects animals in late pregnancy fed low-energy diets and usually occurs during the first or second pregnancy but can be induced in obese virgin females. Ketosis is characterized clinically by rapid onset, inappetence, depression, and death within 2–5 days. Other features include hypoglycemia, lipemia, ketonemia, ketouria, aciduria, and proteinuria. Lesions are nonspecific and include usually a markedly fatty liver and enlarged adrenal glands. Some adrenals have hemorrhages and cortical necrosis (Navia and Hunt, 1976).

Because ketosis usually affects females in last 2–3 weeks of pregnancy, it is often considered the metabolic form of pregnancy toxemia. Both the circulatory and metabolic forms of pregnancy

toxemia are manifested by the same clinical signs of depression, acidosis, ketosis, proteinuria, keto-nuria, and a lowered urinary pH (Percy and Barthold, 2001).

Starvation and Water Deprivation

Starvation and water deprivation should not be underestimated as causes of death in guinea pigs. They are notoriously fixed in their eating habits and do not readily adapt to diets different from those to which they are accustomed. Likewise, they are quite inadept at finding new sources of drinking water in their environment (Wagner, 1979).

Other Diseases, Spontaneous

Alpha-mannosidosis is a lysosomal storage disorder resulting from the deficient activity of lyso-somal alpha-mannosidase. The affected guinea pigs have neural and visceral changes that closely resemble the human disease and will provide a convenient model for investigation of new thera-peutic strategies for neuronal storage diseases, such as enzyme replacement and gene replacement therapies (Crawley et al., 1999).

Bacterial, Viral, and Rickettsial Diseases

Guinea pigs scatter their bedding into feeders and water crocks. They are notorious for chew-ing on and otherwise blocking sipper-tubed waterers. They mix dry feed and water in their mouths and pass the slurry into the sipper tube, thereby blocking the tube or causing it to drip. They will also defecate into their feed and water crocks if the feeders and crocks are not suspended above the bedding (Harkness and Wagner, 1983). Because of these behavioral characteristics, infectious agents can become problems in research studies. Good general husbandry and disease prevention procedures are essential when using guinea pigs.

Bacterial Diseases

Infections, Spontaneous

Guinea pigs are susceptible to a wide variety of microorganisms. At least 19 genera of bacte-ria, mycoplasmas, and rickettsia-like agents have been recovered from laboratory guinea pigs with spontaneous diseases. The most frequently reported bacterial infections causing epizootic disease were *Salmonella* spp., *B. bronchiseptica., Streptococcus* spp. (Lancefield group C), and *Y. pseudotuberculosis.* These infections have been associated often with high mortality in the guinea pig colony. Less frequently reported causes of epizootic disease have been *S. pneumoniae*, *K. pneumoniae*, *P. multocida*, *Actinobacillus* spp., and *Pseudomonas* spp. After the epizootic dis-ease subsides, carriers persist, resulting in enzootic disease. Predisposing factors that contribute to the severity of the illness or deaths in the colony are poor husbandry practices such as unhealthy sanitation, overcrowding, mixing of animal species in the same room, improper ventilation, incorrect temperature and humidity control, inadequate diet, transporting the guinea pigs, and experimental procedures (Ganaway, 1976; Boot et al., 1983).

Because they are susceptible to *Bordetella pneumonia*, guinea pigs should not be housed with rabbits, cats, dogs, and other species that carry *Bordetella* subclinically (Harkness and Wagner, 1983). *Lawsonia* (*Campylobacter*) *jejuni* was isolated from the intestines of guinea pigs with no clinical disease (Meanger and Marshall, 1989).

Infections, Induced

Salmonella typhi introduced into the gallbladder was recovered from bile and feces of infected, but apparently normal, animals of the NIH Hartley strain for up to 5 months. These animals provide a model for the asymptomatic human typhoid carrier (Lavergne et al., 1977).

Experimental infection of guinea pigs with pathogenic *Serpulina* (*Treponema*) *hyodysenteriae* may use as an animal model for swine dysentery (Joens et al., 1978, 1993).

A guinea pig model of low-dose aerogenic tubercular infections has been developed (Chambers et al., 2001). Guinea pigs infected with the Legionnaires' disease bacillus, *L. pneumophila*, have been used to evaluate new therapeutic agents (Edelstein et al., 2001, 2003).

Viral, Rickettsial, and Chlamydial Diseases

Infections, Spontaneous

Evidence of more than 16 viral and chlamydial agents has been reported in guinea pigs. These agents belong to a variety of groups including enterovirus (poliovirus), reovirus, paramyxovirus (Sendai virus, simian virus, parainfluenza type 1), leukovirus (leukemia), arenavirus (lymphocytic choriomeningitis virus), herpesvirus, pox virus, and psittacosis-lymphogranuloma venereum-trachoma (*Chlamydia*). Serological tests revealed antibodies against poliovirus (16%), reovirus type 3 (4%), pneumonia virus mice (23%), simian virus (25%), and Sendai virus (9%). Leukemia, lymphocytic choriomeningitis, cytomegalovirus (salivary gland), guinea pig herpes-like virus (Connelly et al., 1987), guinea pig pox-like virus, and guinea pig inclusion conjunctivitis chlamydophia, *C. caviae*, formerly termed *C. psittaci* (Eidson, 2002), have been isolated and described. Viruses are suspected to be involved in myositis (Saunders, 1958) wasting disease, hepatoenteritis, and pneumonia. Viral, rickettsial, and chlamydial diseases and their agents are described in more detail by Van Hoosier and Robinette (1976).

Infections, Induced

In addition to the naturally occurring or spontaneous diseases, the guinea pig has been proven to be a valuable experimental animal in the study of a wide variety of viral, rickettsial, and chlamydial diseases (Van Hoosier and Robinette, 1976). An example is the adaptation of Pichinde virus, an arenavirus, to inbred strain 13 guinea pigs to produce infections similar by virological, pathological, and clinical features of the human disease Lassa fever. Infections by this arenavirus are a potentially useful animal model of human disease (Moe and Jahrling, 1984; Lucia et al., 1989, 1990). Guinea pigs infected with guinea pig cytomegalovirus develop a mononucleosis syndrome that is similar to that seen in immunocompetent humans (Lucia et al., 1985). Genital herpesvirus infections in guinea pigs are useful models of primary and recurrent infections for studies of prophylactic, antiviral, and vaccine therapies (Stanberry et al., 1985; Simms et al., 2000; Bernstein et al., 2001; Bourne et al., 2002)

They are also susceptible to rickettsial infections, and a number of rickettsiae pathogenic to humans were first studied in the guinea pig (Fenner, 1986). Guinea pigs are susceptible to the Q fever agent *C. burnetii* and provide an animal model of this disease (Heggers et al., 1975).

Mycotic Diseases

The vast majority of spontaneous fungal diseases in guinea pigs are dermatophytoses limited to the skin. Systemic mycoses such as caused by *Mucor* spp. and *Absidia* spp. are sporadic and described in detail by Sprouse (1976). A case of dermal cryptococcosis caused by *C. neoformans* involved the nose (Van Herck et al., 1988).

Parasitic (Protozoan and Metazoan) Diseases

Protozoan Infection, Spontaneous

Although a large number of protozoan parasites are observed in guinea pigs, protozoan diseases are rare. The most important protozoan diseases are intestinal coccidosis caused by *E. caviae* and renal coccidosis caused by *K. cobayae* (Vetterling, 1976). Three protozoa can cause generalized infections during their acute phases: *T. gondii*, the tissue form of the feline coccidia; *Sarcocystis caviae*; and *E.* (*Nosema*) *cuniculi*. Toxoplasma infections may produce encephalitis, visceral granulomas, lymphadenitis, splenomegaly, or myocarditis (Wallach and Boever, 1983). Sarcocystis is found in skeletal muscle, and *Encephalitozoon* has been reported in the brain (Yost, 1958). Protozoan parasites of guinea pigs have been reviewed in detail by Vetterling (1976).

Protozoan Infection, Induced

Guinea pigs were intradermally infected with *Trypanosoma cruzi*, the infective agent for Chagas' disease to evaluate the role of epidermal Langerhans' cells in skin-related immunological events (Nargis et al., 2001). Experimental immunization against *T. cruzi* has been studied in guinea pigs exposed to infected Triatoma bugs (Basombrio et al., 1997). Experimental cryptosporidiosis in guinea pigs is a useful small animal model of this disease (Chrisp et al., 1990).

Metazoan Infection, Spontaneous

The guinea pig may become infested with several different arthropod ectoparasites. Most of these are specific for the guinea pig and well adapted to their host with minimal disease (Ronald and Wagner, 1976).

Metazoan Infection, Induced

Experimental infections by the fluke *Fascioloides magna* have demonstrated that the guinea pig is a suitable animal model for studying this parasite, an important pathogen in sheep (Conboy and Stromberg, 1991; Conboy et al., 1991).

The only helminth of importance reported in guinea pigs is the cecal worm *P. uncinata*. This intestinal nematode rarely causes clinical disease (Wescott, 1976).

Immunological Disease

Spontaneous Conditions

An inbred strain of complement 4-deficiency guinea pigs has a total deficiency of the C4 component of complement with no evidence of a C4 inhibitor in the serum (Ellman and Green, 1970). Passive anaphylaxis, contact and delayed hypersensitivity, and cellular exudative response to a foreign body are normal indicating the presence of an alternate complement pathway (Ellman et al., 1971a; Frank et al., 1971). The NIH Hartley strain of guinea pig is susceptible to acute systemic anaphylaxis (Stone et al., 1964). Amyloidosis of multiple organs, particularly the kidneys, liver, spleen, and adrenal glands is rather commonly found in guinea pigs (Wagner, 1976). Guinea pigs deficient in the fourth component of complement did not have glomerulonephritis (Foltz et al., 1994). Athymic or hypothymic hairless guinea pigs are agammaglobulinemic and may be used for studies of immunodeficiency diseases (Reed and O'Donoghue, 1979).

Induced Conditions

Strain 2 guinea pigs develop an immune response to bovine serum albumin, 2,4-D protamine, human serum albumin, potassium dichromate, beryllium fluoride, hydralazine, insulin, human fibrinopeptide B β1–14, GA, DNP-PLL (2,4,dinitrophenol-polylyzine), PLL, and DNP-PLA (2,4,dinitrophenol-polylactic acid) (Ellman and Green, 1971; Ellman et al., 1971b; Alspaugh and Van Hoosier, 1973; Barcinski and Rosenthal, 1977; Geczy and DeWeck, 1977; Rose et al., 1979; Thomas et al., 1979a,b). Basement membrane from lung and kidney readily evoke antibody production (Milgrom et al., 1979), forming the basis of some commercial antibody production enterprises.

Strain 13 guinea pigs develop an immune response to benzylpenicilloyl bovine γ-globulin, DNP guinea pig skin protein conjugates, 2,4-DNP guinea pig albumin, mercuric chloride, aspirin, poly (TGAG), GT, insulin, and human fibrinopeptide B β1–14 (Geczy and DeWeck, 1977).

The sensitivity of the respiratory system of the guinea pig has provided animal models for numerous studies of inhalation phenomena, including bronchospasms; asthma; latex allergy (Aamir et al., 1996) and hypersensitivity reactions to dust, 2,4-toluene diisocyanate (Sugawara et al., 1993), and other air pollutants; and development of antihistamines.

CONCLUSIONS

The guinea pig has great usefulness as an animal model in toxicology for a broad variety of experimental diseases and test systems in several organ systems. There are relatively few infectious, nutritional, and metabolic diseases that might interfere with toxicology studies. The majority of spontaneous diseases is well described and can be controlled by appropriate husbandry practices. More information on husbandry, pathology, and diseases, including treatment, are given in the works of Melby and Altman (1974), Wagner and Manning (1979), Benirschke et al. (1978), Hime and O'Donoghue (1979), Wallach and Boever (1983), Rowsell (1984), Manning et al. (1984), Fraser et al. (1986), Anderson (1987), Poole (1987), Harkness and Wagner (1983), Richardson (2000), and Percy and Barthold (2001); and necropsy of the guinea pig is described by Feldman and Seely (1988). The detailed anatomy is presented by Cooper and Schiller (1975).

METABOLISM

The objective behind the use of any animal model in toxicology is almost always to predict what will happen in humans. A necessary and essential part of this is knowing the reasons for any differences in response between the model and the human and being able to scale these between the species (Remmer and Merker, 1963; Cazen, 1987; Nakayama et al., 1987). A major component of such differences is undoubtedly variations (qualitative and quantitative) in metabolism (Thabrew and Emerole, 1983; Smith et al., 1986; Souhaili-El Amri et al., 1986; Neal et al., 1987). CYP-450 isoform activities remain not well characterized in guinea pigs (Kibler et al., 1947; Boutin et al., 1984; Astrom et al., 1986, 1987). Lue and Chan (2000), Oecsh et al. (1988), and Patoine et al. (2013) have characterized the induction of CYP1A1 with frying oil and Fernandez (2001) has published mainly on lipid metabolism in the species. Metabolism of voriconazole, which is primarily by CYP3A4 in humans, rats, and dogs, is minimal and not inducible in the guinea pig (Roffey et al., 2003). Successfully identified in the guinea pig have been CYP1A1, 1A2, 2E1, 3A, 3A14, 3A15, 4A13, 11B1, 11B2, and 17 (Abe and Watanabe, 1982; Peterson et al., 1983; Liu and Chan, 2000; Izumi et al., 2006; Hewitta et al., 2010; Patoine et al., 2013).

Table 5.14 summarizes some key factors about guinea pig metabolism of xenobiotics.

Table 5.14 Major Metabolic Characteristics of the Guinea Pig

Characteristics	References
Highly dependent on adequate dietary ascorbic acid	Sikic et al. (1977)
Has higher sulfotransferase and glucuronosyltranferase form 1 in the jejunum than in the liver.	Hietanen and Vainio (1973); Hayakawa et al. (1977); Schwenk and Locher (1985)
Has higher substrate specificity for monooxygenases (benzo[*a*]pyrene hydroxylase and *N*-demethylase) in intestines than in liver.	Gregus et al. (1983, 1985) Laitinen and Watkins (1986)
Liver size is proportionately (to body size) greater than in the human (2% of total body weight in the human, 3.5% in the guinea pig).	Calabrese (1988); Feldman and Seely (1988)
Lipoprotein metabolism and its association with inflammation are similar to that in humans.	Fernandez and Volek (2006)
Does not have aromatic hydroxylation capacity for amphetamines.	Caldwell (1981)
While rat and dog have more efficient biliary excretion than man, the guinea pig is comparable to man.	Levine (1978)
Significant adrenal metabolism that exhibits in mature animals	Pitrolo et al. (1979)

The guinea pig is showing value as a broad model of metabolic diseases. CYP3A expressions can be decreased to provide a model of diet induced metabolic syndrome. Hyperlipemia can be used to induce triglyceride metabolism disorders (Fernandez and Volek, 2006; Yang et al., 2011).

Cardiac glycosides readily evoke antibodies the pharmacokinetics of which are comparative to those in humans (Chung et al., 1982). The guinea pig also depends significantly on metabolism by its gut flora (Zannoni and Lynch, 1973; Wong, 1976; Ton and Fong, 1984; Rowland et al., 1986).

Species Peculiarities

As is the case with all other animal models that have been studied for some time, a number of responses to potentially toxic xenobiotics have been identified in the guinea pig that is different from humans or other model species (Quinn et al., 1958; Caldwell, 1982). This section will seek to overview these differences.

Guinea pigs are remarkable in their requirements for vitamin C. The animal maintained on a vitamin C–deficient diet develops scurvy-like symptoms. Before the chemical methods were developed, guinea pigs were used for vitamin C assay in nutritional studies. The guinea pig is highly susceptible to human and bovine tuberculosis, and it may be used for the diagnosis of human mycobacteriosis. The guinea pig is an excellent model for anaphylaxis and other immunological procedures, but this excellence is founded on the fact that the immune responses in the guinea pig (both cellular and humoral) are exaggerated compared to humans.

Antibiotic Toxicity

Guinea pigs are highly sensitive to antibiotics, particularly those specific for gram-positive organisms (Boyd and Fulford, 1961; Melnikona and Rodionov, 1979). The normal intestinal flora of guinea pigs is predominantly gram positive. Administration of antibiotics specific for gram-positive bacteria destroys the normal flora of the intestinal tract and permits an overgrowth of gram-negative organisms. Until recently, complications following antibiotic administration were attributed primarily to *E. coli*, but current evidence suggests that *C. difficile* plays an important role in the enterotoxemia that follows antibiotic treatment. These antibiotics are not directly toxic and are not harmful when administered in therapeutic dosages to germfree animals. Tetracycline, cephaloridine, chloramphenicol, and the sulfonamides are among the less hazardous antimicrobials, but all should be administered with caution, using the minimal effective dosage.

Response to Anti-Inflammatories

The area of anti-inflammatories is (and had been for at least 30 years) a very active one in pharmaceutical research. These efforts have been hampered by the lack of good models for assessing the safety of such compounds, particularly in terms of potential gastrointestinal and renal toxicity.

Many of the anti-inflammatories (particularly the nonsteroidal anti-inflammatory drugs [NSAIDs]) act at least in part by inhibiting prostaglandin synthesis (Steel et al., 1979; Steffenrud, 1986). Some of the standard models used for screening for potentially effective compounds include (1) assaying homogenates of the stomach of dosed guinea pigs for prostaglandin synthesis and (2) inhibition of UV-induced erythema in the skin of dosed guinea pigs (Birnbaum et al., 1982).

The guinea pig, though still more sensitive than the human to the gastrointestinal ulceration induced by NSAIDs, is less so than the dog or rat for a range of NSAIDs (Wilhelmi, 1974; Mariani and Bonanomi, 1978; Birnbaum et al., 1982) making it an attractive model for studying gastroprotective agents (Schinatarelli et al., 1984). The most common toxic effect reported for anti-inflammatories in the guinea pigs is gastrointestinal ulceration, however, and they are generally more sensitive than humans.

Cardiotoxic Effects of Catecholamines

Waldenstrom et al. (1987) have performed extensive evaluations of the cardiotoxicity of catecholamines in both the rat and guinea pig and have found the guinea pig to be less sensitive than the rat to the local anoxia-caused damage induced by catecholamines.

Strains

There are three naturally occurring strains of guinea pigs, which can be distinguished from each other by differences in the length, texture, and direction of their hair growth. These are the (1) short-haired, smooth, and coarse-coated English; (2) the long-haired Abyssinian, which has its hair arranged in whirls; and (3) the long, fine-haired Peruvian.

The English guinea pig, which comes as an albino, bi-, or tricolored animals and as outbred and inbred strains, is the commonest and the most often strain used in the laboratory. They are alert and well fleshed with smooth, shiny skin. The albino Hartley strain is the variety most familiar to toxicologists, and it is considered the standard animal for skin sensitization studies.

Recently, Charles River Breeding Laboratories has developed and made commercially available a hairless form of the Hartley guinea pig, designated the Crl:IAF/HA(hr/hr)BR). This is a fertile, euthymic animal. There are also athymic/hypothymic varieties available.

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The Rabbit

Clare M. Salamon and Shayne Cox Gad

CONTENTS

TOXICOLOGY

Clare M. Salamon

History

The domestic rabbit, *Oryctolagus cuniculus*, which belongs to the order Lagomorpha (and hence is referred to as a lagomorph) and the family Leporidae (which also includes hares), is descended from the wild rabbits of western Europe and northwestern Africa, which were first discovered by the Phoenicians in 1100 BC (Fox, 1974). The rabbit is one of the most successful mammals of the world; it is both prolific and adaptable and appears to be equally at home on all the continents. Domestic rabbits are similar to rodents in many respects. The principal anatomical difference is that rabbits have two pairs of upper incisor teeth, whereas rodents have only one pair (Flatt, 1977). The additional pair of incisors in rabbits is smaller and located directly behind the other pair.

Domestication of the rabbit probably began in monasteries during the sixteenth century (Fox, 1974). By the middle of the seventeenth century, the rabbit was completely domesticated and rabbit raising was active in England and continental Europe. This is the only species of lagomorph that has been domesticated, and as such, the species has been introduced into every country of the world as a source of meat or fur, as a pet, or as a laboratory animal.

Early descriptions of research with rabbits date back to 1683 when Leeuwenhoek first described the dominance of the wild-type coat ("normal" white-bellied agouti) over albinism, nonagouti, etc. (Sirks, 1959). Since then, rabbits have been used extensively to investigate the genetics of coat color and hair morphology, and the rabbit is one of the most common species used for biomedical experimentation. In addition, their by-products are utilized for pharmaceutical and vaccine production.

Choice of the Rabbit in Toxicological Research

Justification for Use

A number of size, shape, and color variations derived from centuries of selective breeding constitute more than 50 well-established breeds recognized by the rabbit breeders' associations. Representatives of the small breeds (under 2 kg or 5 lb) include the American Dutch and Polish breeds, the medium-sized breeds (2–5 kg or 5–10 lb) are the California and New Zealand White (albino) rabbits, and the large breeds (5 kg or 10 lb and over) are the Flemish and Checkered Giants.

The New Zealand White albino is the rabbit most commonly used for research purposes and is the focus for this chapter. However, other breeds, such as the American Dutch, Flemish Giant, and Polish, are also used as laboratory animals.

Compared to the high cost of cats, dogs, and monkeys and the problems associated with their proper care and maintenance, rabbits are relatively inexpensive, hardy, small, clean, and more easily housed and handled. Thus, they are readily used for a wide variety of experimental procedures and testing situations, including immunology (they are good antibody producers and their blood is relatively easy to collect from their large and accessible ear veins), teratological, dermal, ocular, and implant studies. Specific study designs are discussed later in this chapter and are outlined in Tables 6.1 through 6.4.

Because the distribution of intestinal microflora within the gut (and hence their potential metabolic activity) is more similar in rabbits to that of humans than that of the guinea pig, rat, or mouse (Williams, 1972), an orally administered compound is less likely to be metabolized, possibly into a toxic or active metabolite, in the rabbit than in these other laboratory species. However, since gastric emptying time for the rabbit can vary anywhere from 20 min to 20 hours in contrast to the human rate of 1.0–1.5 hours (Smyth and Hottendorf, 1980), the rabbit is a poor species for evaluating the absorption of orally administered compounds (Maeda et al., 1977).

Table 6.1   Selected Normative Data for New Zealand White Rabbits

General adult body weight	
Male	4–5 kg
Female	4–6 kg
Life span	5–13 years
Surface area	2.5 kg, 1270 cm^2
	4.8 kg, 3040 cm^2
Chromosome number (diploid)	44
Water consumption	50–100 mg/kg/day
Food consumption	50 g/kg/day
Rectal temperature	102.0–103.5°F
Basal metabolism	110 cal/kg/day
GI transit time	4–5 h
Reproduction	
Puberty	
Breeding age	3–8 months
Male	6–10 months
Female	5–9 months
Breeding season	All year (controlled conditions)
Estrus cycle	Polyestrous, induced
Breeding habits	Female brought to male
Time of ovulation	9.75–13.5 h; postcopulation
Time of implantation	Approx. 7 days; postcopulation
Length of gestation	29–35 days
Postpartum estrus	None
Litter size	4–10
Birth weight	30–100 g
Weight at 3 days	400–700 g
Weaning age	4–6 weeks
Weaning weight	1–2 kg

Table 6.2   Minimum Cage Sizes

Weight		Floor Area/Rabbit	
kg	lb	ft²	m²
<2	5	1.5	0.14
2–4	5–10	3.0	0.28
4–5.3	10–12	4.0	0.37
≥5.4	≥12	5.0	0.46

Source: Courtesy of ILAR (1985).
Note: The cage height should be at least 14 in. (25.56 cm).

Although rabbits are frequently used to study dermal toxicity, they may not be the best species. Because human skin has a thicker stratum corneum, it is more resistant to the dermal absorption of foreign substances and is penetrated much less easily by xenobiotics than the skin of the most widely used animal models, including the rabbit and the rat (Calabrese, 1984). In vitro, skin permeability to ionic and covalent substances in aqueous solution and organic solutes increases in the following order: human, pig, guinea pig, rat, and rabbit (Tregear, 1966). When Bartek et al. (1972) compared the in vivo skin permeability of several compounds, they also found that the absolute dermal absorption rate for the rabbit was higher than that of the rat, pig, or human (in a descending order). It appears that because the permeability of the skin of miniature swine is close to that of human skin, for studies in which dermal toxicity data are to be used to predict toxicity in humans, in some respects, the miniature swine appears to be a more suitable test animal than the rabbit. However, despite the considerable species difference between rabbit and human skin, rabbits are routinely employed in dermal toxicity tests. Thus, when interpreting data generated using rabbits, the researcher must consider the following (Bartek et al., 1972). In general, for a single dermal dose, total exposure of the outer surface of the skin to the applied compound will be of shorter duration in the rabbit than in humans. However, because of the higher penetration rate in the rabbit, temporarily higher concentrations of compound might occur within the rabbit skin as compared to human skin. In addition, the time course of systemic exposure to the compound will also be markedly different in the two species.

Important Physical and Physiological Characteristics

Some general values for selected physical and physiological data in rabbits are in Table 6.1. More detailed information on these characteristics may be found under the appropriate subsection in this chapter.

Growth and Development

The New Zealand White rabbit grows very rapidly. At weaning, these animals weigh 1.0–1.5 kg and the adult animal weighs 4–6 kg depending on sex. The life span of most strains of rabbits is 5–8 years, and some may live upto 15 years (Harkness et al., 2010). One index of age is the size and appearance of the claws. They do not project beyond the fur until the rabbit approaches maturity, and then they grow and curl with age.

Reproduction

The age at which rabbits reach puberty varies from 4 to 12 months and depends somewhat on breed and strain. It tends to be inversely related to size, i.e., smaller rabbits like Polish rabbits may be bred at 4 months, whereas heavier Flemish rabbits reach puberty between 9 and 12 months.

Table 6.3   Nutrient Requirements[a]

Energy and protein	
Digestible energy (kcal)	2100–2500
TDN (%)	55–65
Crude fiber (%)	16–20
Fat (%)	2–4
Crude protein (%)	14–18
Inorganic nutrients	
Calcium (%)	0.4–0.5
Phosphorus	0.22–0.4
Magnesium (mg)	300–400
Potassium	0.6
Sodium (%)	0.2–0.5
Chloride (%)	0.5
Copper (mg)	3–10
Iodine (mg)	0.2
Iron (mg)	100
Manganese (mg)	8.5–40
Zinc (mg)	50
Vitamins	
Vitamin A (mg)	0.33–0.44
Vitamin A as carotene (mg)	0.83
Vitamin E (mg)	20–40
Vitamin K3 **(1119)**	2
Niacin (mg)	180
Pyridoxine (mg)	39
Choline (g)	1.2[b]
Amino acids	
Lysine	0.65
Methionine + cystine	0.6
Arginine	0.6
Histidine	0.3[b]
Leucine	1.1[b]
Isoleucine	0.6[b]
Phenylalanine + tyrosine	1.1[b]
Threonine	0.6[a]
Tryptophan	0.2[b]
Valine	0.7[b]
Glycine	—[c]

Source: Data from Clarke, H. E. et al., *Lab. Anim. Sci.*, 11, 1, 1977; Hunt, C. E. and
Harrington, D. D., Nutrition and nutritional diseases of the rabbit, in *The Biology
of the Laboratory Rabbit*, Weisbroth, S. H., Flatt, R. E., and Kraus, A. L., eds.,
Academic Press, New York, 1974, pp. 287–315; National Academy of Sciences,
Nutrient Requirements of Rabbits, 2nd edn. rev., Washington, DC, 1977.
[a] Presented as percent (%) or mg/kg in the diet.
[b] May not be minimum but known to be adequate.
[c] Quantitative requirement not determined, but dietary need demonstrated.

The New Zealand White rabbit may be bred at 5–6 months (female) and 7–8 months (male). The male rabbit matures slower than the female. Motile spermatozoa appear in the ejaculate at 4 months, and adult levels of sperm production are reached at about 7 or 8 months (Bivin and Timmons, 1974).

Although rabbits do not have a definite estrus cycle, there are short periods of 1–2 days every 2 weeks when the doe is not receptive to the male. Also, a doe may refuse to mate with one buck but may be receptive to another. Mating behavior consists of tail flagging and enurination, and

Table 6.4 Chemical Composition of Purina Certified Rabbit Diet (A) and Purina Certified High-Fiber Rabbit Diet (B)

Nutrients	A	B
Energy and protein		
Protein, min. (%)	16.0	14.0
Fat, min. (%)	2.5	1.5
Fiber, max. (%)	18.0	25.0
Moisture, max.	12.0	—
Ash, max. (%)	8.0	10.0
Added minerals, max. (%)	2.1	1.5
Nutrients		
Protein (%)	16.2	14.5
Fat (%)	2.5	1.7
Cholesterol (ppm)	61.0	60.0
Fiber, crude (%)	13.0	22.5
Neutral detergent fiber	27.4	40.9
Acid detergent fiber 950	15.8	24.6
Total digestible nutrients	66.0	57.0
Nitrogen-free extract, by difference	52.0	42.4
M		
Gross energy (kcal/g)	4.0	3.9
Physiological fuel (kcal/g)	2.95	2.43
Ash (%)	7.3	8.9
Calcium (%)	0.95	1.2
Phosphorus	0.50	0.5
Potassium (%)	1.15	1.67
Magnesium	0.25	0.30
Sodium	0.25	0.32
Chlorine	0.50	0.79
Iron (ppm)	276.2	315.9
Zinc (ppm)	105.5	122.2
Manganese (ppm)	107.2	127.5
Copper (ppm)	27.8	25.8
Cobalt (ppm)	0.38	0.46
Iodine (ppm)	0.59	0.58
Chromium (ppm)	4.5	5.0
Selenium (ppm)	0.13	0.2
Vitamins		
Vitamin A (IU/g)	20.0	20.0
Carotene (ppm)	27.6	31.2
Vitamin D (IU/g)	2.3	2.2
Ci-tocopherol (IU/kg)	44.0	33.0
Niacin (ppm)	33.0	33.4
Pyridoxine (ppm)	4.5	4.5
Pantothenic acid (ppm)	19.0	19.1
Thiamine (ppm)	3.5	2.8
Riboflavin (ppm)	5.0	8.6
Choline (ppm × 100)	16.0	16.0

(Continued)

Table 6.4 (*Continued*)   Chemical Composition of Purina Certified Rabbit Diet (A) and Purina Certified High-Fiber Rabbit Diet (B)

Nutrients	A	B
Folic acid (ppm)	2.0	3.3
Biotin (ppm)	0.12	0.14
B_{12} (IUg/kg)	6.6	6.6
Amino acids		
Lysine	0.78	0.61
Methionine	0.35	0.30
Arginine	0.90	0.68
Histidine	0.40	0.32
Leucine	1.3	1.06
Isoleucine	0.82	0.79
Phenylalanine	0.80	0.65
Tyrosine	0.50	0.42
Threonine	0.64	0.56
Tryptophan	0.23	0.19
Valine	0.84	0.68
Glycine	0.77	0.70
Cystine	0.25	0.20

Source: Courtesy of Ralston Purina Co., St. Louis, MO.

sometimes chasing. In tail flagging, the buck elevates his hindquarters, walks stiff legged, and lays his tail flat on his back, providing visual stimulus to the doe and olfactory stimulus from the inguinal glands. In enurination, the buck may turn his hindquarters toward the doe and emit a jet of urine in a display of sexual aggressiveness; sometimes, this is followed by a circling of the cage. Enurination may also be directed toward bucks in adjacent cages. If the doe is receptive, she will elevate her hind quarters, permitting the buck to mount.

Rabbits are induced ovulators, which means the female remains in estrus until copulation, which triggers the induction of ovulation. Ovulation can also be induced by an injection of chorionic gonadotropin or other luteinizing hormones, which is done if the researcher wishes to employ artificial insemination. Ovulation occurs 10–13 hours after copulation or after the injection of luteinizing hormone.

Rabbits have been used extensively for reproductive studies because of the precise timing that can be obtained for studies of egg maturation, fertilization, cleavage, and implantation. Pregnancy can be confirmed by palpation approximately 10–12 days after breeding. Fertile copulations result in pregnancy and a gestation of 30–35 days depending on the breed. Parturition is called kindling.

Clinical signs of pregnancy or pseudopregnancy are an increase in the size of the ruff, a large tuft of hair on the ventral neck, and a subsequent increase in hair pulling, especially from the ruff to make a nest.

Pseudopregnancy, which lasts 16–17 days, is easily induced in the doe and can be caused by infertile mating, sexual excitement from a doe mounting another doe, injection of luteinizing hormone, or stress due to shipping or experimentation. During pseudopregnancy the doe is not receptive to mating.

Excretion

Rabbits have two types of feces: soft, moist nighttime feces and firm, dry daytime fecal pellets. The nighttime feces, which are covered with mucus and consist primarily of secretion from

the cecum, are produced by the initial ingestion of food; hard feces are produced by reingestion of the soft feces. The soft feces are protein and vitamin rich, and their ingestion (coprophagy) directly from the anus improves the utilization of nitrogen (Thacker and Brandt, 1955), provides an abundance of certain B vitamins, plays an important role in the incorporation of sulfur in the soft tissues, and conserves water (Kulwich et al., 1953, 1954). Wild rabbits are able to survive up to a week without food because of reingestion of feces.

Because of their diet, rabbit urine is cloudy and ranges in color from light yellow to deep orange or red brown and may resemble purulent discharge. It contains carbonate and phosphate crystals, which cause a scale to accumulate on cage surfaces.

Husbandry

As with other laboratory animals, the facilities, equipment, and husbandry procedure used for rabbits should be designed to afford maximum environmental control (i.e., minimal variation in temperature, humidity, and ventilation); optimal conditions for that animal's comfort, health, and welfare; and minimal exposure to injury and disease.

Facilities

Rabbits should be maintained in an area that is clean and dry, with adequate ventilation, away from excessive noise, and within a specified range of temperature and humidity.

Temperature, Relative Humidity, and Ventilation

Because the thermal environment can influence the severity, duration, and variability of toxic responses to chemical by influencing the physiology of the animal and the metabolism and disposition of test materials (Fuhrman and Fuhrman, 1961; Weihe, 1973; Clough, 1982), the temperature, humidity, and ventilation of the animal facilities must be strictly controlled (Rao, 1986). The thermoneutral zone of resting laboratory animals (and humans) is very small (Weihe, 1973). When exposed to temperatures outside their thermoneutral zone, laboratory animals adapt behaviorally (unless they are prevented from doing so by experimentally enforced restrictions) and/or metabolically (by increasing or decreasing their metabolic rates). Thus, marked variations in the environmental conditions of the animal room may alter the animal's rate of metabolism and, ultimately, the potential toxicity of a test material.

Ambient relative humidity is also a major factor in maintaining the heat balance of an animal (Clough, 1982). It is a very important consideration with dermal absorption studies, particularly when shaved animals are used. In these situations, the relative humidity will directly affect the rate of evaporation, the viscosity of the test material, and the animal's peripheral circulation, thus having a major impact on the potential toxicity of the applied material.

For rabbits, animal room temperatures should be maintained between 60 and 70°F (16 and 20°C) and relative humidity should be between 40 and 60% (ILAR, 1996). This temperature range is lower (vs. 64.4–78.8 or 84.2 and 18–26 or 29°C) and the relative humidity range is narrower (vs. 30%–70%) than that required for other common laboratory animals (ILAR, 1996). With rabbits, elevated temperatures in particular can result in lower male fertility, lower conception rates for the does, and reduced litter sizes.

The recommended ventilation rate is 12 cu ft/min/animal (up to approximately 5.6 kg)/day (Runkle, 1964) or at least 10 complete air changes/hour (ILAR, 1996), preferably with 100% fresh air. Recirculation of air is not recommended. If the air is recirculated, more efficient filtration will be required to remove odors and contaminants.

Light and Noise

Most facilities maintain light cycles of 12 hours light and 12 hours dark. However, light cycles of 14–16 hours light for females are recommended when the rabbits are used in reproductive studies.

Situations that might result in elevated noise levels for extended periods of time, e.g., housing rabbits in the same area as dogs or in the vicinity of noisy equipment, should be regarded with caution and avoided if possible. Nayfield and Besch (1981) have reported indications of stress, i.e., increased spontaneous activity and adrenal weights and decreased spleen and thymus weights in rabbits that were exposed to elevated noise (1.5 hours of white noise at intensities of 107–112 dbls/ day for 2 weeks). An auditory stimulus also appears to affect plasma cholesterol concentrations (Friedman et al., 1967) and initiates changes in the hypothalamus (Henkin and Knigge, 1963) of exposed rabbits.

Caging

Rabbit cages and racks should be constructed of a smooth, corrosion-resistant material that is impervious to liquids and moisture, preferably stainless steel, and is easily sanitized and sterilized. The floor of the cage is usually made of steel grid or wire mesh with a recommended size of 1 in. × ½ in. or ⅝ in.²/1 × 2.5 cm. It should be smooth and free of sharp projections. The wire mesh floors allow excrement to fall to excreta trays below the cage. Some types of welded wire (1 in. × 1 in. or 1 in. × 2 in.) or solid metal walls with air vents are generally used on the dies; back and top of the cage and wire grid is used on the front. Painting of cages and racks is not recommended. The racks and cages should be movable to facilitate transportation to a washing area.

The amount of floor space required/animal (or the cage size for individually housed animals) is determined by the weight of the rabbit. Current minimum space recommendations for rabbits are in Table 6.2 (ILAR, 1996). It is recommended that the racks holding the cages be placed at least 2 m apart to minimize the between-rack spread of airborne microorganisms due to convection currents (Teelman and Weihe, 1974).

Two standard sizes of cages that are available commercially and provide 3 and 4 ft² of floor area have dimensions (width × depth × height) of 24 in. × 18 in. × 16 in. and 24 in. × 24 in. × 15 in., respectively. Young immature rabbits may be group housed by sex, but rabbits that are sexually mature (over 4 months) often attack one another and should always be individually housed. Because wounding, pseudopregnancies, and infertility may occur in groups housed together, mature rabbits should be paired only at mating.

Nest boxes must be provided for does that are expected to deliver and nurse young. Suitable bedding used in nest boxes should be a nonedible material, e.g., wood shavings.

Dropping pans are lined with disposable absorbent liners that should be changed at least 3 times each week. The animals should be transferred to clean cages at least every 2 weeks. Facilities should be physically cleaned and sanitized at least 3 times a week.

Rabbit urine, with a pH of 8.2, is very alkaline and contains phosphate and carbonate crystals that accumulate on the cage surfaces and form a scale that is difficult to remove. Detergents, disinfectants, and lime-scale removers (acidic solutions such as vinegar or acid products at pH 2) may be applied with a stiff brush in routine cage cleaning. However, acidic materials, which may cause discoloration and damage the cages, should be used with caution. Flaming may also be necessary to remove hair and manure and kill coccidial oocysts.

Feed and Water

Feed and water systems should be clean and designed so that they cannot become easily contaminated. Metal feeders that attach to the front of the cage and can be filled outside without

opening the cage door are commercially available and should be used in preference to crocks or other open containers. Likewise, water bottles (if an automatic water system is not available) are usually mounted on the outside on the front of the cage. Sipper-tube watering devices are preferred.

Rabbits should be provided ad libitum with a plentiful supply of fresh, clean water. Nonpregnant does drink approximately 10 mL/100 g of body weight per day, and lactating does may drink up to 90 mL/100 g/day (Harkness et al., 2010). If water bottles are used, they should be filled with fresh water daily and sanitized at least once weekly.

Food hoppers should be constructed of durable material (other than wood) that is resistant to the gnawing of rabbits, is corrosion resistant, and is easily sanitized. They should be mounted 4 in. from the floor of the cage, and the design should permit easy access, but not allow the rabbit to enter. They should also be free of sharp edges that could cause cuts or scratches.

The preferred diet is a wholesome, nutritious, pelleted form of feed, free of drugs, hormones, pesticides, and animal and vermin contaminants. Because of the great variability in fiber and nutrient content in commercially available diets (Wise and Gilburt, 1980, 1981) and because of the potential presence of contaminants, it is recommended that only diets that have been analyzed for these materials be used. Recommended nutrient levels are presented in Table 6.3. The nutrient content of two commercially available diets, Purina Rabbit Diet and Purina Certified High-Fiber Rabbit Diet (PMI® Nutrition International), which are made of natural ingredients and purified, is presented in Table 6.4.

Fresh feed should be provided at least weekly. However, a once-daily feeding (limited feeding) of approximately 120 g (4 oz or ⅔ cup) of pellets is sufficient to maintain an adult, medium-sized rabbit at a constant weight; this is the amount of feed that may be given to animals that are to be used for acute studies. For longer studies, the animals are provided with approximately 190 g (6 oz or 1 cup) of pellets each day. At peak lactation, a doe may consume up to 450 g (16 oz) of feed per day, and pregnant or lactating does should be provided with feed ad libitum. The feeders should be checked daily and any powdered feed removed.

The diet should contain 16%–20% crude fiber and 14%–18% crude protein (Harkness et al., 2010). Rabbits have a higher requirement for fiber than other species. Fiber has an important role as bulk, but rabbits do not digest over 18% dietary fiber in a single passage. Diarrhea may result if the fiber concentration is below 6%, and fiber above 20% may lead to lowered feed efficiency (Harkness, 1987). The feeding of diets containing a high concentration of fiber (18%–20%) is often utilized to reduce the occurrence of intestinal enteropathies.

Malnutrition is uncommon in rabbits. Other relatively uncommon nutritional problems include vitamin D, calcium, and phosphorus imbalances (atherosclerosis), vitamin A (hydrocephalus, prenatal death) and vitamin E (muscular dystrophy, prenatal mortality, seminiferous tubule degeneration) deficiencies, and some specific mineral or amino acid deficiencies.

Because feed and bedding are generally used in animal facilities without prior sanitization, they are potential sources of contamination for diseases, parasites, and hormones. Therefore, potential problems could occur and caution should be used in purchasing and storing food and bedding.

Handling and Restraint

Rabbits must be picked up and held correctly to prevent both animal and human injuries. They should be handled firmly but gently. Because they are very shy animals and are easily frightened, they may often struggle and try to escape. With the exception of older bucks and primiparous does with strong territorial instincts, rabbits seldom bite people.

However, a rabbit that is picked up incorrectly or is not held securely will kick violently with its hind legs, and it is very easy to cause injury to the rabbit, particularly a broken back. In addition, while trying to escape, rabbits can inflict painful scratches on the handler with their powerful hind legs.

Rabbit ears are very fragile and they should never be used to pick up the animal or as a means for restraint. A safe method to handle or pick up rabbits is by firmly grasping the loose skin at the base of the skull (this area is commonly called the nape or the scruff) with one hand while supporting the rear legs with the other hand. A rabbit can also be held by encircling its body with one arm, with the sternum supported by the hand, while holding the scruff with the other hand. Or it can be held with one arm holding the rabbit's hindquarters and pressing the animal toward the handler's body. The abdomen and sternum are then supported by the handler's forearm, with the other hand on the scruff. If the hindquarters are not supported, the rabbit may struggle. If the rabbit does struggle, it can be easily calmed by placing it on the forearm with the head concealed in the bend of the handler's elbow.

Various types of restraining devices are available for use with rabbits. They serve primarily to control the body of the rabbit while the head or ears are being manipulated. The most common devices are usually some variation of a restraint box or stocks. If the basic stocks/restraining boxes are used, the rabbit may need to be trained and calmed to avoid struggling, which may cause a broken back. A squeeze-cage stock is often used for intravenous injections, and a cat bag may be used for gavage administration. The rabbit can also be manually restrained. If manual restraint is used, the rabbit should not be placed directly on a smooth surface because it may flail. Instead, it should be placed on a towel or mat.

Dosing Techniques

Compounds may be administered to rabbits by a variety of routes. The more commonly used routes and specific techniques are described as follows.

Oral Administration

The simplest method of administering compounds orally is by incorporating them in the feed or water. However, as with other species, rabbits may not voluntarily consume the material if it has an unpleasant odor or taste or if it is in a form that cannot be easily consumed. In addition, rabbits tend to spread their feed around and "play" with their water bottles. Thus, because of potential palatability problems, spillage, and wastage, these methods may not be satisfactory if it is important that precise amounts of the material be administered.

Very small volumes of liquid materials may be administered by placing the tip of a ball-tipped syringe in the corner of the rabbit's mouth and slowly introducing the material. However, the most accurate method of administering compounds orally is to deliver the material by gavage. This may be done using a stainless steel ball-tipped needle (13-gauge) or a latex catheter (size 14 French, 16 in. long). The needle is attached to a syringe with a locking end, the head and neck of the animal are manually restrained to avoid injury if the animal should struggle, and the needle is inserted into the back of the mouth and into the esophagus.

When using a latex catheter for gavage, the animal is also restrained. The animal may be manually restrained or a cat bag can be used for restraint. The mouth is held open and the catheter is inserted into the back of the throat, being careful to avoid the teeth, and then gently introduced into the esophagus and into the stomach. To establish that the catheter is in the stomach and not the bronchi, there should be no air passage in the tube that corresponds to respiratory movements. If the animal struggles, the catheter should be removed and reinserted. After the catheter is inserted, a ball-tipped needle that is attached to a syringe is inserted into the open end of the catheter, and the plunger of the syringe is gently pushed to administer the dose. Depending on the nature of the material and the dose volume, it may be advisable to flush the catheter by leaving the catheter in place and affixing another syringe with water or the appropriate vehicle. The catheter, attached to the empty syringe, is then removed, again being careful to avoid the teeth. The volume of material administered

is based on the vehicle used. In general, rabbits should not be given more than 2 mL/kg of corn oil; water or a water-based solution may be given at volumes up to 6 mL/kg.

Dermal Administration

Materials are applied topically on the dorsal area of the trunk. The fur is removed with an electric clipper before dosing and as needed thereafter. Care should be taken to avoid abrading the skin and only animals with healthy, intact skin should be used. If the dosing material is a liquid, it may be applied diluted or undiluted. If the dosing material is a solid, it should be slightly moistened with saline, deionized water, or another suitable vehicle before application to ensure good contact with the skin. The dosing material is then applied uniformly over the exposure area (from approximately 5 cm^2 to an area estimated to constitute approximately 20% of the total body surface area). The exposure area may be left uncovered or it may be held in contact with the skin by a gauze dressing secured with tape, covered with cellophane wrap, and overwrapped with elastic tape. The animals should be fitted with flexible "Elizabethan"-type plastic collars to prevent them from removing the dosing material or coverings (Barnett, 1958). At the end of the treatment period, the bindings and collar are removed and the exposure area may be washed with water and/or wiped clean with a towel moistened with water or a mild soap solution as thoroughly as possible without irritating the skin. The volume of material given is based on the size of the animal and the area of skin that is available for application.

Ocular Administration

Animals should be examined before the administration of the dose material, and any materials that show preexisting ocular defects, irritation, or corneal injury should not be used. If a solid or granular material is to be administered, it should be finely ground into a dust or powder. The material is placed in the conjunctival sac, and the upper and lower lids are then gently held together for a second before releasing to prevent loss of the dose material. The amount given should not exceed 10 μL or the equivalent weight.

Intravenous Administration

Materials are usually administered intravenously into the marginal ear vein (along the posterior edge on the outside of the ear). The animal is restrained, the hair is gently plucked from the area of the vein, and a disinfectant is applied. If the vein is not readily apparent, the area can be rubbed with alcohol, or the vein can be occluded by gently pressing on the base of the ear or by placing a paper clip at the base of the ear. For intravenous injections, the needle should be sharp (prepackaged, sterile, disposable needles are the best) and should be the smallest size possible (1 in., 25-gauge, or smaller). The needle should first be inserted into the skin beside and parallel to the blood vessel and then inserted into the vein with the beveled edge up. The syringe should only contain the dosing solution and no air. If there are signs of engorgement or swelling at the injection site, indicating that the needle is outside the vein, reposition the needle. After the material has been injected, withdraw the needle, and apply digital pressure to the puncture site for a short time to prevent bleeding.

Subcutaneous Administration

Subcutaneous injections are made under the skin of the neck or back, using a 1 in., 23–27-gauge needle. The skin is grasped with one hand and the material is injected into the tented skin. If large volumes are to be injected, the needle can be withdrawn slightly and redirected to spread the fluid over a wider area.

Intramuscular Administration

Intramuscular injections are made into the lateral aspects of the large muscles in the hindquarters, e.g., the gluteal or thigh muscles. Care must be taken to avoid hitting large blood vessels, nerves, and bone. First, the area of the injection site is immobilized and cleaned with disinfectant, and the needle is then inserted ½–¾ in. perpendicularly, depending on the muscle thickness. After the needle is inserted, the plunger of the syringe should be withdrawn slightly to confirm that no blood can be aspirated; i.e., the needle has not inadvertently entered a blood vessel. If blood appears in the syringe, the needle should be carefully repositioned or withdrawn and reinserted. After the needle is placed properly, the fluid is injected slowly and the needle is withdrawn. The smallest gauge needle possible (1 in., 23–27-gauge) should be used for this procedure.

Intradermal Administration

Intradermal injections are given in the loin and flank areas where it is difficult for the rabbit to reach with its hind feet. The hair should first be removed from the site with clippers and depilatory cream. An antiseptic is then applied. The skin is stretched and the needle is inserted only into the dermis, forming a small bleb, and the injection is made using a 25-gauge needle attached to a syringe containing the dosing material. When the needle is removed, the site should be examined for seepage of the dosage material. If this occurs, the needle should be inserted further into the skin during the injection.

Intraperitoneal Administration

The rabbit is manually restrained with the hindquarters elevated, and the needle (⅝ in., 23–25-gauge) is inserted bevel up into an area just lateral to the midline and just posterior to the area of the umbilicus at a steep angle toward the spine. The insertion is made up with a quick thrust through the subcutaneous tissue and ventral abdominal muscles and abdominal wall and into the peritoneal cavity. Using this procedure, up to 20 mL of fluid may be given in a single injection.

Vaginal Administration

The doe is placed in a restraining stock, and the tail is gently grasped between the thumb and forefinger, while the rest of the hand is pressed firmly on the hip region of the animal. As the animal relaxes, the tail is pulled upward to expose the vaginal opening. A catheter (size 8 French, or a ball-tipped stainless steel 1½ in., 18-gauge needle) is gently inserted approximately 1 in. into the vagina, the dose is administered, and the catheter or needle is withdrawn. The animal is kept in the restraining stock for 1–2 min after the procedure is complete to ensure that the dose material is retained.

Collection Techniques

Blood Collection

Ear Vein

Blood is usually collected from the large, readily accessible marginal ear veins. To facilitate collection procedures, the animal is placed in a restraint box that allows access to the ears. A small amount of petroleum jelly is spread along the marginal vein. This causes the hair to lay flat in a natural direction toward the edge of the ear. A disinfectant such as 70% ethyl alcohol is applied and a paper clip may be applied as a tourniquet proximal to the intended bleeding site. Using a lamp as a heat source, the ear is warmed, which causes the vein to become engorged with blood. Slight digital

irritation of the tip of the ear will also increase blood flow and facilitate blood collection. Although xylene is sometimes used for this purpose, it should not be used if the sample is to be used to determine white blood cell counts because of its severe inflammatory properties. While keeping the ear over the lamp, the vein is incised midway between the tourniquet and the tip of the ear, and blood is collected directly in appropriate tubes or containers. Alternatively, a needle attached to a syringe may be used and the blood drawn into the syringe by slow, steady manual pressure.

Auricular Artery

When larger blood samples are required, they may be collected from the centrally located auricular artery using a 20- or 23-gauge needle that is inserted into the artery toward the base of the ear.

Catheterization

Techniques for obtaining multiple blood samples from rabbits over short or extended period of time, which are not traumatic, do not cause hematomas and do not require anesthesia, invasive surgery, or sophisticated expertise that involves implanting a flexible catheter into the auricular artery (Smith et al., 1988; Heim, 1989). The site of the arterial puncture is depilated or the entire ear is shaved. A 2% nitroglycerin ointment may be applied over the artery to prevent arterial spasms, which can interfere with successful catheterization, and the area is stroked with the forefinger until the artery becomes engorged with blood. A 1 in., 22-gauge catheter is inserted into the artery approximately 3 cm from the tip of the ear with the needle at a 25° angle and the bevel up. The appearance of blood in the flash chamber confirms arterial entry. The catheter is then advanced into the arterial lumen until the hub meets the surface of the skin (the hub of the catheter should be below the tip of the ear) and an injection cap is attached to the hub of the catheter. Approximately 0.3 mL of heparinized (5 µ/mL) saline should be injected into the catheter to determine if the placement is correct. This is followed by rapidly infusing an additional 1 mL of heparinized saline and then aspirating blood until adequate blood flow is established. Tissue adhesive may be used to adhere the catheter hub to the skin surface. A piece of rolled gauze is placed inside the ear and then the ear and catheter hub are wrapped with adhesive tape, binding the ears together to prevent the rabbit from removing the injection cap and catheter. For longer sampling periods, gauze or bandaging tape should be used under the adhesive tape to prevent skin irritation.

For all these methods, blood flow from the puncture site is easily stopped by applying firm digital pressure at the site with a gauze pad. Alternatively, after the blood sample is collected, direct pressure may be applied to the incised area and a paper clip applied as a tourniquet to control bleeding until clotting takes place. The use of a surgical lubricant with the paper clip facilitates clotting.

Cardiac Puncture

Cardiac puncture may be used to collect larger quantities of blood or multiple blood samples from anesthetized or sedated rabbits. However, cardiac puncture is traumatic and can result in death. The rabbit should be anesthetized and placed in right lateral recumbency or dorsal recumbency on a restraining board. The heartbeat may be used to directly locate the heart or the needle (1½ in., 18–21-gauge) is inserted at approximately a 30-degree angle immediately behind the xiphoid cartilage.

Posterior Vena Cava/Iliac Bifurcation

When large volumes of blood are needed at terminal sacrifice and known enzyme alterations caused by cell trauma from other bleeding techniques are contraindicated, blood may be obtained from the posterior vena cava or iliac bifurcation of anesthetized animals.

The animal is placed on its back and a midline incision is made that exposes the abdominal viscera. The large intestine is moved laterally, away from the inguinal area, exposing the large abdominal vessels. The iliac vein is surgically isolated (cranial) just above the bifurcation of the internal iliac and femoral veins. If the posterior vena cava is to be used, it is isolated in a similar manner, just cranial to the common iliac vein and caudal to the renal vein. Because this is a terminal sampling procedure, a sterile surgical technique does not apply. Venipuncture is made using an appropriately sized flexible catheter and needle (the size depends on the size of the animal but will generally be between 20 and 25-gauge). After the needle is inserted into the vein, the flexible catheter is slid forward past the tip of the needle, further into the vein, and the needle is withdrawn. Blood will well up in the hub of the catheter and is collected using the syringe. Any bleeding around the venipuncture site may be controlled by digital pressure with a piece of gauze or cotton. Alternatively, a ligature can be positioned around the vessel before venipuncture and then firmly tied after the needle is inserted.

To obtain a blood sample, the injection cap and catheter are flushed with heparinized saline; approximately 0.3 mL is then aspirated to clear the saline from the catheter, and the required amount of blood is withdrawn. Patency is maintained by flushing the catheter and injection cap with a higher concentration of heparinized (100 μ/mL) saline and then leaving them filled with the solution between sampling intervals.

This technique has been used successfully to collect as many as eight blood samples from a single rabbit within a 2 hours period (with sampling intervals as short as 5 min) and for infusion periods that have lasted as long as 7 days (patency was maintained by daily flushing with heparinized saline). It is particularly applicable for studies to establish the pharmacokinetic properties of new drugs and for observing the reaction of rabbits to drug administration. It can also be used to obtain large (greater than 30 mL) amounts of blood.

Urine Collection

The most common method used to collect urine is by placing the animals in stainless steel metabolic cages, where the urine and feces are separated by a cone-shaped device (the urine drains off the collecting walls into a tube and the feces drop into an inverted cone). Food and water are provided in such a way that the urine will not be contaminated. However, some hair and fecal contamination of the urine may occur.

If contamination of the sample with fecal and other materials is not acceptable, urine samples may be obtained by urethral catheterization (practicable in males only) or by direct puncture of the bladder (cystocentesis).

Catheterization

For this procedure, the catheters and any lubricating gels and speculums must be sterilized before use. The animal is sedated or anesthetized and restrained in dorsal recumbency, and the penis is extruded. The catheter (commercially available cat urethral catheter) is introduced into the urethral opening and gently advanced into the urethra and then the bladder. If the catheter is left partially in its container, it can be held without becoming contaminated. When urine flow begins, a syringe can be attached to the catheter and used to aspirate the urine remaining in the bladder.

Cystocentesis

For urine collection directly from the bladder through the body wall, the animal should be sedated and the skin shaved in the midline in the inguinal region. The bladder, located by palpation

in the posterior abdomen, is held firmly through the body wall, and the needle (1 or 1½ in., 23-gauge) is introduced through the body wall in the midline at approximately a 45° angle. The needle should be inserted in the posterior section of the bladder so it will remain in the bladder lumen as the urine is withdrawn. The urine may be expelled through the needle into a collection tube or by attaching a syringe to the needle.

Special Procedure

Anesthesia

Because there is a great deal of interrabbit variability in sensitivity to anesthetics and because there is a narrow margin between anesthesia and death, a great deal of caution should be exercised in situations requiring anesthetization. In addition, the frequent presence of preexisting lung damage related to infection with *Pasteurella multocida* may cause respiratory failure during a period of anesthesia. Recovery from anesthesia in rabbits is often slow, particularly following the use of barbiturates, and the prolonged inappetence that is often a postoperative complication can result in gastrointestinal disturbances.

For routine anesthesia of rabbits, Flecknell (1996) recommends fentanyl/fluanisone (Hypnorm) (0.3 mL/kg intramuscular) and midazolam or diazepam (2 mg/kg intramuscular, intravenous, or intraperitoneal). This drug combination provides good surgical anesthesia with excellent muscle relaxation for 20–40 min. It is recommended that fentanyl/fluanisone be administered first, followed 10–15 min later by midazolam or diazepam. Longer period of anesthesia can be achieved by administering additional doses of Hypnorm (approximately 0.2 mg/kg intravenously every 30–40 min). See Flecknell (1996) for a complete discussion of the use of preanesthetic and anesthetic medication in rabbits.

The depth of anesthesia should be monitored by rate and depth of respiration and degree of jaw tension. Other indicators such as pedal reflexes, corneal reflex, and papillary size are unreliable.

Euthanasia

Euthanasia may be accomplished by using an overdose of a barbiturate such as sodium pentobarbital or a commercial euthanasia solution such as T-61.

Reproduction Procedures

To determine the sex of adult rabbits, the skin is gently pressed back from the genital opening. In males, the penis will be everted; females have an elongated vulva with a short slit-like opening. The mature male also has perineal, or inguinal, pouches lateral and anterior to the penis. In prepuberal rabbits, when pressure is applied against the genital orifice, the penis everts equally all the way around, whereas the vulva protrudes only laterally and ventrally; the posterior end that does not evert is attached near the anus.

Natural Mating

When breeding rabbits naturally, the doe must be taken to the male's cage. If the female is receptive, copulation will occur soon after introduction to the male. The buck mounts the doe and grasps the female's body with a foreleg on each flank. Intromission is usually accomplished after 8 to 12 rapid copulatory movements, and ejaculation follows on the first intromission. Immediately after ejaculation, the male may fall backward or off the side (both hind feet are off the ground during ejaculation) and emit a cry.

Artificial Insemination

For artificial insemination procedures, semen is collected in an artificial vagina. The buck is introduced to a teaser doe. When the buck begins to mount, the vagina is positioned so that the buck can ejaculate into it. Semen samples show considerable variation between individuals for the same individual at different times. Therefore, the semen should be evaluated each time it is collected for amount (should have at least 0.5 mL) and color (if yellow/tan, it is probably contaminated with urine and should be discarded). To assess sperm viability, a small amount of semen is pipetted onto a glass slide and the sperms are observed using a 10× objective. Motility is graded as streaming (50%–100% motility), waving, or shaking. Acceptable samples are streaming; waving or shaking samples should be discarded. The sample is then diluted 1:200, and a sperm count is done. The concentration of sperm should be at least of 40×10^6 sperm/mL. The semen preparation should be incubated at 37°C during the insemination procedure and must be used within 30 min of collection time.

Artificial insemination is done using methods similar to those for vaginal dosing. The doe is manually restrained, and the vaginal opening exposed. An insemination pipette (glass tubing approximately 18 cm in length with an inside diameter of 3 mm and an outside diameter of 4 mm) that is bent at 45° angle approximately 4 cm from one end is warmed in a 0.9% saline maintained at 37°C. A rubber bulb is attached to the longer end of the pipette and approximately 0.25 mL of diluted semen is gently drawn into it. The short end of the pipette is gently inserted into the vagina with the tip directed toward the dorsal wall to avoid the urethral orifice. After the pipette has been inserted as far as the angle, it is rotated 180° so that the tip points toward the ventral wall and insertion is continued until slight resistance is felt. The bulb is gently squeezed to expel the semen while the pipette is slowly withdrawn. Ovulation is then induced by giving the doe an injection of 0.1 U/g of human chorionic gonadotropin into the marginal ear vein.

Use in Toxicology Studies

The rabbit is used in numerous study designs to evaluate toxicological responses to pesticides, drugs, and industrial chemicals. Depending on the type of material to be evaluated, these tests are done according to the U.S. Food and Drug Administration, Environmental Protection Agency, Federal Hazardous Substances Act, Department of Transportation, or the Organisation for the Economic Co-operation and Development guidelines and range from short-term acute studies to 90-day toxicity studies.

The rabbit is also the nonrodent species most frequently used to evaluate developmental toxicity. (In the 1960s, the drug thalidomide was tested and shown to be safe in rats, but caused severe birth defects in humans. When given to pregnant rabbits, this drug caused fetal malformations, and now it is required that chemicals must be tested for developmental toxicity in both rodents and nonrodent species.)

Ocular and dermal irritation studies and dermal toxicity studies are types of acute studies that are routinely done using rabbits.

Ocular Irritation

Eye, or ocular, irritation studies generally last 72 hours, but can be continued for 21 days if irritation persists. The guidelines that are followed indicate the number of animals tested and the number of test groups. The rabbits are given an examination to eliminate any animals with preexisting ocular defects, irritation, or corneal injury. The material is instilled in the conjunctival sac and the eye is examined at the specified intervals. If the pH of the test material evaluated is less

than 3.0 or greater than 11.5, consideration should be given to not performing the test, since the material can be considered corrosive.

Dermal Irritation and Toxicity

Acute dermal irritation and toxicity studies last 14 days. The length of exposure is 24 hours, and the numbers of animals and dose levels depend on the specific testing guidelines. Clinical observations and dermal irritation scores are recorded at specified intervals. As with the ocular study, if the pH of the material is less than 3.0 or greater than 11.5, consideration should be given to not performing the test, since the test material can be considered corrosive.

Dermal Toxicity

The rabbit is also used to evaluate the dermal toxicity of a test material. This study may be a short-term, 14-day study or may be up to 90 days in duration. Clinical observations, body weights, and food consumptions are recorded weekly, and dermal irritation scores are recorded daily. The animals are necropsied and microscopic examinations are done. Animals are bled before the terminal sacrifice and hematology and clinical chemistry data are obtained.

Teratogenicity

Rabbits are frequently used as the nonrodent species for teratogenicity studies. In this study design, the pregnant doe is treated during fetal organogenesis, days 7–19 of gestation. The day of breeding or artificial insemination is gestation day 0. The doe is euthanized prior to term (gestation day 29), and the uterine contents are examined for implantation sites, early and late resorption sites, and live or dead fetuses. The fetuses are weighed, sexed, and examined for external, soft-tissue, and skeletal development.

Parasites, Diseases, and Physical Anomalies

Because it is very difficult and expensive to obtain pathogen-free rabbits, the identification of common diseases and parasites is important to the investigator. In some instances, these conditions may cause an animal to be unsuitable for a study or may require euthanasia of a specific animal or an entire shipment of animals. This section includes a brief discussion of some of the more frequent conditions observed with rabbits, common clinical signs to aid in identification, and suggested treatments.

Mites

Ear Mites

Ear mange (otitis externa) is a condition caused by psoroptid mites that are nonburrowing parasites that chew the epidermal layers of the skin and produce a tan or brown crusty exudate on the inner surface of the ear. Clinical signs are the rabbit shaking its head or scratching its ears. Although ear mites are nonpathogenic, they can spread from rabbit to rabbit and, if left untreated, can result in a secondary bacterial infection. The treatment is simple: the ear canal should be cleansed and treated with a few drops of plain mineral oil or mineral oil with a miticide added. A high standard of hygiene should be maintained (Kraus, 1974).

Fur Mites

There are two types of fur mites that are commonly seen in laboratory rabbits: the cheyletid and sarcoptid mites. The cheyletid fur mite is seen mainly on the dorsal trunk in the scapular area. Often, there are no clinical signs except for partial alopecia and a grayish white skin surface; generally, there is no scratching by the rabbit. This mite is nonburrowing and ingests the keratin layer of the epidermis. Treatment for this mite consists of the application of a topical gel acaricide (Holmes, 1984).

Mange Mites

Another mite that can be seen in groups of laboratory rabbits is the sarcoptid mange mite. Unlike the noninvasive cheyletid fur mite, the sarcoptid mite tunnels through the skin, ingests epithelial cells, and may suck lymph. Clinical signs are rubbing and alopecia and a whitish yellow crust. The rubbing may lead to skin lesions and secondary bacterial infections. This condition is contagious and can become severe, leading to emaciation and death (Holmes, 1984). The recommended treatment is elimination of affected animals and clean, sanitary conditions.

Protozoan Infections

Coccidiosis

One of the most important diseases in rabbits is coccidiosis, which is caused by protozoan organisms of the genus *Eimeria*. There are two major types of coccidiosis that are detrimental to rabbits: one affects the liver and the other affects the intestine. Transmission is by ingestion of sporulated oocysts that are in the feces (Harkness, 1987).

Encephalitozoonosis

Encephalitozoonosis is a chronic disease seen in rabbits and is caused by the protozoan *Encephalitozoon cuniculi*. It is usually latent and actually prevalent in many colonies (estimates range from 15% to 78% affected). However, it is extremely difficult to diagnose because the only clinical signs are occasional neurological signs such as convulsions, tremors, and torticollis (Harkness, 1987). Transmission is thought to be via infectious urine. Pathologically, lesions are noted in the kidney and the brain. There is no known treatment (Holmes, 1984). The disease is important because it may interfere with and complicate the interpretation of experimental data.

Hepatic Coccidiosis

Eimeria stiedae is the organism that affects the liver. Clinical signs such as diarrhea and weight loss are often seen only in young animals; adults usually show no physical change. Pathologically, this organism affects the bile duct epithelium and causes an enlarged or irregularly shaped liver (Pakes, 1974).

Intestinal Coccidiosis

Eimeria irresidua, *E. magna*, *E. media*, and *E. perforans* are the organisms responsible for intestinal coccidiosis. Clinical signs vary depending on the extent of infection and the age of the animal. In cases of mild infections, there are no clinical signs; severe infections result in weight loss, diarrhea, dehydration, and secondary bacterial infections (Holmes, 1984). Prevention and control of coccidiosis is by strict sanitization practices and elimination of infected animals.

Bacterial Infections

Pasteurella multocida

P. multocida is a frequent cause of bacterial disease in rabbits. It causes a multitude of clinical diseases such as snuffles, pneumonia, otitis media, abscesses and conjunctivitis, metritis, or pyometra and orchitis. Many rabbits carry the organism without any clinical signs of infection until they are stressed as a result of experimental procedures.

Snuffles. This disease is also referred to as rhinitis or sinusitis. It occurs when *P. multocida* is found in nasal cavities (Flatt, 1974). It is quite common for rabbits to have this infection without any clinical signs. When stressed, sneezing, coughing, and a nasal discharge may develop. The disease may persist as snuffles or it may progress to other clinical forms.

Pneumonia. P. multocida may cause pneumonia in young rabbits 4–6 weeks of age. It can progress to cause consolidation in the lungs or pleuritis or, in the case of acute pneumonia, may result in death without any clinical signs (Holmes, 1984).

Septicemia. Septicemia has often been associated with snuffles and pneumonia. Clinical signs for septicemia are not observed because, once infected, the animal dies quickly (Holmes, 1984).

Otitis. P. multocida may spread from the nasal cavities to the inner ear and cause torticollis or otitis interna (Holmes, 1984). This should not be confused with otitis externa, which is caused by mites. Rather, it is an infection of the inner ear that is often characterized by a head tilt or wry neck and may be so severe that the head is upside down.

Metritis and orchitis. Another condition caused by *P. multocida* is metritis, or pyometra, in the doe and orchitis or epididymitis in the buck. Genital infections occur more in the doe, although they also are observed in young rabbits and adult bucks. Clinical signs of the acute or subacute infection in the doe are seldom seen except for a vaginal discharge. The condition can cause a reduction in fertility either by a failure of the does to conceive or by the possible enlargement of the testes of the bucks (Flatt, 1974).

Abscesses and conjunctivitis. These conditions are frequently seen in rabbits that have been exposed to *P. multocida* (Flatt, 1974). The abscess swellings contain a thick, tan or white creamy exudate and may be surrounded by a fibrous capsule. Conjunctivitis, possibly caused by the bacteria entering the conjunctival sac via the nasolacrimal duct, may occur in both young and mature rabbits. Clinical signs of conjunctivitis are reddened conjunctivae, moderately swollen or closed eyelids, and a mucus exudate (Flatt, 1974).

P. multocida is easily transmitted from rabbit to rabbit. It can be spread from dam to young by the respiratory route. It can have venereal transmission when genital infections are present. Since rabbits are infected with the disease without any clinical signs, it can be transmitted when new stocks are introduced into an established colony. The bacteria are sensitive to a number of antibiotics such as chlortetracycline, oxytetracycline, penicillin, ampicillin, and chloramphenicol (Holmes, 1984). However, rarely do these treatments provide a complete cure. More often, remission occurs and infection is reestablished when treatment is ceased or when the rabbit is stressed. The best cure is elimination of the disease by euthanasia of affected animals and stringent sanitation practices. Also, it is recommended that investigators purchase rabbits from commercial colonies that provide rabbits that were cesarian derived and maintained in isolation (Holmes, 1984).

Tyzzer's Disease

Bacillus piliformis causes a bacterial disease commonly referred to as Tyzzer's disease. There is not a lot of information available on Tyzzer's disease; however, it is thought to be widespread. It is often diagnosed as mucoid enteritis because of the watery diarrhea, stained hindquarters, and dehydration that are seen clinically and very few animals survive (Flatt, 1974). This disease generally

affects young rabbits, 10–12 weeks of age, but can occur in adults and preweanlings. It is transmitted through ingestion of feces of infected animals. Then, if the rabbit is stressed because of overcrowding, heat, or experimentation, the organisms multiply. Treatment of infected animals rarely results in complete elimination of the bacteria. The best remedy is to eliminate infected animals and apply good sanitation practices (Holmes, 1984).

Staphylococcal Infections

There are little data concerning the incidences of staphylococcal infections; however, *Staphylococcus aureus* is the most commonly identified pathogen. Clinical identification of the disease is difficult and is dependent on the site and duration of infection. These infections may range from dermatitis and slight subcutaneous swellings to septicemia and death (Flatt, 1974). Staphylococcal infections can be treated with systemic antibiotics, but they are best controlled by maintaining the animals in a clean, sanitary environment.

One of the more common staphylococcal infections is mastitis, or "blue breast." It is most prevalent in does that are heavy milk producers or that have sustained an injury and are maintained in unsanitary conditions. Clinical signs are fever, decreased appetite, and inflamed mammary glands. These infections are not limited to lactating does and may also be seen in pseudopregnant does that are maintained in unsanitary conditions (Holmes, 1984). The disease is contagious and infected does should be isolated.

Escherichia coli

Clinical signs of *Escherichia coli* infections are similar to other bacterial infections, i.e., diarrhea and death. This type of infection is thought to be a result of changes in the intestinal flora due to changes in environmental conditions. Although this organism is the predominant bacteria cultured, it is also thought to be a secondary infection rather than the primary cause of death (Flatt, 1974).

Treponematosis

Treponematosis, or rabbit syphilis, is characterized by inflammation of the face, genitalia, or regional lymph nodes. It is caused by a bacterium *Treponema cuniculi*. Although it is not commonly seen clinically, the disease is often observed serologically. Treponematosis has been confused with hutch burn, and the lesion may look like that seen with ear mites or sarcoptic mange. The lesions are primarily seen on the external genitalia, but they are also found on the chin, lips, nose, and eyelids. Treponematosis is transmitted by direct contact and is easily treated by isolation of infected individuals and penicillin (Flatt, 1974).

Mucoid Enteropathy

Mucoid enteropathy is probably one of the most common diseases affecting rabbits and at the same time is the least understood. It has been called bloat, enteritis, mucoid enteritis, mucoid diarrhea, and scours. It is most prevalent among young rabbits, 7–10 weeks of age, and is associated with the change in nutrition and feed of the suckling rabbit and the weanling. It is not certain if mucoid enteropathy is a disease in itself or just occurs in conjunction with other diseases. It is one of the most common causes of death in young rabbits. Clinical signs are diarrhea, depressed food consumption, hypothermia, bloated abdomen, depression, hunched posture, and the perineal region stained with yellow- to brown-colored fecal material. Macroscopic examinations frequently show stomach and sections of the intestines distended with gas or a watery fluid, gelatinous contents in

the colon, and noninflammatory lesions in the intestinal mucosa. Microscopic examinations of the intestinal lesions support the noninflammatory condition and indicate an increase in the production of goblet cells (Flatt et al., 1974). The most productive treatment of mucoid enteropathy is management by prevention, practice of good sanitation, reduction of stress, and elimination of animals with clinical signs. Maintaining the animals on restricted feed rations is sometimes effective, or if that is not possible, restricting feed for the first few days postarrival and then gradually increasing to ad libitum. Also, feeding a high-fiber diet has been shown to be effective in reducing the incidence of mucoid enteropathy.

Ulcerative Pododermatitis

Ulcerative pododermatitis is often seen among groups of older rabbits that have been maintained in wire cages for long periods of time. It is a pressure necrosis of the skin and is considered the result of heavy body weight on wire cage floors. It has mistakenly been called sore hocks, but this is an inaccurate term, since the affected area is the entire metatarsal region and is not restricted to the hocks (Flatt et al., 1974). The lesions vary in size and are well-defined, ulcerated areas in the skin that are covered by a crusty scab. Secondary bacterial infections and abscesses may occur in the adjacent dermis (Holmes, 1984). The affected animal may appear clinically healthy, or it may show a weight loss, hunched appearance, and shifting of weight. The lesion can be treated and the reoccurrence reduced by housing the animal in solid-bottom cages with soft bedding material.

Fractures

The rabbit skeleton is very fragile and is only about 8% of its body weight (compared to a cat skeleton, which is 13% of its body weight) (Harkness et al., 2010). Because the long bones and lumbar spine are surrounded by powerful muscle masses, the rabbit is particularly susceptible to fractures of the legs and back. Thus, although not a disease, the identification and diagnosis of the severity of a traumatic vertebral fracture is important. It is a posterior paralysis resulting from a vertebral fracture and damage to the spinal cord (Flatt et al., 1974). The onset is sudden, and often the fracture is the result of a struggle during restraint or improper support of the hindquarters when handled. Diagnosis is by clinical examination or radiography. Depending on the location of the lesion and the severity of the fracture, clinical signs are motor paralysis, loss of control of the anal sphincter and urinary bladder, and edema at the site. After a few weeks, function may return and the rabbit may not show any obvious signs of a previous injury (Flatt et al., 1974). However, if the injury is serious enough, it may be necessary to euthanatize the animal.

Moist Dermatitis

Moist dermatitis, also known as sore dewlap or hutch burn, is the result of constant wetting of the fur. It is a chronic, progressive disease and there may be various degrees of bacterial involvement. It may be caused from drooling because of malocclusion; drinking from open pans; cold, damp contact bedding; or continuous wetting of the fur because of experimental design (Holmes, 1984). Treatment is by the elimination or reduction of the causes, i.e., by using water bottles and sipper tubes or automatic watering systems instead of open pans, by observing good sanitation, and by assuring that the fur is kept clean and dry.

Trichobezoar

Hair that has been ingested can accumulate in the stomach in the form of a ball or trichobezoar. It generally does not cause any problems and is only noted at necropsy unless the mass becomes

very large in size and obstruction can occur. If without apparent reason an animal suddenly stops eating and drinking, a hair ball might be the cause. Hair balls can be detected by palpitation or radiography. Successful treatment consists of giving mineral oil by a stomach tube if the hair ball is not too large.

Buphthalmia

Congenital or infantile glaucoma, buphthalmia, is a relatively common disease in laboratory rabbits. It is an increase in the size of the anterior chamber of the eye and may progress to cloudiness in the cornea, flattening of the cornea, and increased prominence of the eyeball. The condition progressively worsens over time and there is no treatment (Lindsey and Fox, 1974).

Heat Prostration

Rabbits are very sensitive to heat and husbandry practices should assure controlled temperatures. Clinical signs of heat prostration include rapid respiration, cyanosis, and prostration. Does near kindling and young rabbits in nesting boxes are particularly prone to temperature extremes. Hyperthermia can also cause abortion and a reduction of male fertility.

Malocclusion

Another clinical finding that is not necessarily life threatening but could have an adverse effect on an investigation is malocclusion. Rabbit teeth grow continuously and malocclusions occur when the incisors do not properly occlude and, therefore, are not worn away. Causes could either be a genetic shortening of the upper jaw or the result of an injury. This condition can be easily managed by clipping the teeth on a regular basis.

PATHOLOGY

Clare M. Salamon

The previous section presented some of the most common diseases with their treatment that may be encountered in laboratory rabbits during toxicology studies. This section on pathology provides additional information on these diseases as well as spontaneous gross and microscopic changes seen in the common diseases. Some less common conditions of laboratory rabbits will also be discussed. The occurrence of spontaneous diseases must be considered when interpreting the outcome of toxicology experiments (Baker, 1998; Baker, 2003). During some experimental studies, changes will be observed that are incidental and have no effect on the results. In other studies, the experiments will exacerbate the severity or incidence of common findings (Dahlgren, 1992). The material is presented by organ system with a brief review of diseases primarily affecting that system, their gross and microscopic anatomy, and some of the associated spontaneous pathology.

Integumentary System

Skin

The rabbit body is covered with an abundant fine curly undercoat and stiffer guard hairs. The skin has a well-differentiated superficial fascia composed of elastic and dense collagen. Numerous blood vessels lie immediately under the dermis. These features facilitate subcutaneous injections

into folds of skin over the back, shoulder, and neck regions. The ears are highly vascular and have prominent marginal veins that can be used for venipuncture. The neck has a large fold of skin called the dewlap that is predisposed to moisture accumulation and moist dermatitis (see below).

Dermal irritation studies and acute dermal toxicity studies are routinely done using rabbits (Anderson and Henck, 1994). In these studies, test materials are applied to the skin. Afterward, depending on the experimental protocol, the sites of application are observed at defined time periods for possible adverse effects. Frequent gross observations range from erythema to ulcer formation. The severity of these lesions depends upon the duration and frequency of application in addition to the chemical properties of the material involved. Microscopically, these lesions range from mild hyperemia and edema to extensive ulceration. Rabbits were more sensitive than human subjects when exposed to similar amounts of isopropylmyristate (Campbell and Bruce, 1981). Griffith and Buehler (1981) compared the responses of skin from rabbits to those from guinea pigs and humans to primary irritants. Rabbit skin reacted with greater severities than guinea pigs or human. Guinea pig reactions were more predictive of the human response to primary irritants than those of rabbits.

Alopecia, the loss or thinning of the hair, is most often observed on the head or back. It can be the result of ectoparasite infestations involving a variety of mites, mycotic infections such as dermatomycosis or "ringworm," and hair chewing or "barbering" by cage-mates. Alopecia is more common in old rabbits. Alopecia was associated with ribavirin administration in rabbits (Gillett et al., 1990).

Otitis externa is a manifestation of the ear mite, *Psoroptes cuniculi*, the most common and costliest ectoparasite in domestic rabbits (Suckow and Douglas, 1997). These are obligate, nonburrowing mites that chew the epidermal layers of the skin and produce marked inflammation. Affected rabbits are often observed shaking their heads or scratching their ears. In heavily parasitized ears, foul-smelling branlike tan or brown crusty exudates may fill the inner surface of the external ear. The ear is often thickened and edematous.

Alopecia and dermatitis of the face, nose, lips, and external genitalia have been associated with *Sarcoptes scabiei*, *Spilopsyllus cuniculi*, and *Notoedres cati*. Unlike the noninvasive fur mites, these mites tunnel through the skin, ingest epithelial cells, and may suck lymph. Early skin changes are alopecia with a whitish yellow crust. Common clinical signs of sarcoptic infestations are pruritus including intense rubbing and scratching that can lead to severe skin lesions and secondary bacterial infections, followed by anemia, leucopenia, self-mutilation, general debility, emaciation, and death (Percy and Barthold, 2001). Notoedres infestations are less common and usually mild (Hofing and Kraus, 1994).

Fur mites, *Cheyletiella parasitovorax*, can be present with little clinical evidence or a mild alopecia with a grayish white skin surface that affects the back especially over the shoulders. These mites are nonburrowing and ingest the keratin layer of the epidermis. Areas of variable degrees of hair loss, hyperemia, scaliness, and crusts are present. Microscopically, these mites cause a mild subchronic dermatitis with hyperkeratosis, congestion, and inflammatory cell infiltrations. These inflammatory cells may include polymorphonuclear neutrophils, mononuclear cell phagocytes, lymphocytes, plasma cells, and small numbers of eosinophils (Foxx and Ewing, 1969). This alopecia is accompanied with a pruritus that is only evident after careful observation. Generally, there is no scratching by affected rabbits. Another fur mite, *Listrophorus gibbus*, has been reported sporadically in domestic rabbits. This mite may be more common than currently realized because it causes no clinical signs of its presence. The preferred site of attachment is the underside of the tail and with little movement in the hair (Hofing and Kraus, 1994). See the section "Toxicology" for treatment of ectoparasites.

Morgan et al. (1985) described treatment-related skin lesions of epidermal necrosis, hyperkeratosis, and inflammation during skin irritation tests that became infected by dermal mucormycosis. *Rhizopus* sp. was identified in the tissues. The development of fungal dissemination may require more time than the usual 7–21-day studies.

Dermal mycotic infections such as dermatomycosis or "ringworm" are uncommon. They may be subclinical or characterized by raised, circumscribed, erythematous areas with crusted surfaces and hair loss. Lesions usually occur on the head, face, and ears with less frequent secondary spread to the forepaws. They can be very pruritic with affected rabbits scratching vigorously at lesions. The most common cause is *Trichophyton mentagrophytes*. Less commonly, *Microsporum canis* is involved. Microscopically, characteristic changes include hyperkeratosis, epidermal hyperplasia, and folliculitis with mononuclear and polymorphonuclear cell infiltration. Fungal arthrospores in infected hair shafts are demonstrated by methenamine silver and PAS stains.

Hair chewing or "barbering" is occasionally observed in young, group-housed rabbits. It occurs on the face and back as a patchy alopecia with no evidence of a concurrent dermatitis. Contributing factors of boredom and low-roughage diets have been implicated. Abrasions with hair loss are common as a result of fighting by group-housed rabbits that have reached sexual maturity. The lesions after fighting include lacerations around the external genitalia. Aggressive males may injure both bucks and does, thereby resulting in skin abrasions and amputation of the tips of the ears.

Hair pulling can result in patchy thinning or hair loss in adult rabbits especially in nest-building females as well as boredom or seasonal and idiopathic moulting.

Exfoliative dermatosis and sebaceous adenitis have been reported, primarily in older adult pet rabbits. It is characterized as a nonpruritic scaling dermatosis with patchy to coalescing areas of alopecia. Microscopic changes include hyperkeratosis, follicular interface dermatitis, interface folliculitis, reduction in the numbers of sebaceous glands with destruction and lymphocytic infiltration, and perifollicular to diffuse dermal fibrosis (White et al., 2000).

Ulcerative dermatitis or pododermatitis (sore hocks) is often seen among groups of older rabbits that have been maintained in wire cages for long periods of time. The condition is commonly termed sore hocks, but this is inaccurate, since the affected area is the entire metatarsal region and is not restricted to the hocks. Affected animals may appear clinically healthy or may show a weight loss, hunched appearance, shifting of body weight, or reluctance to move. Lesions occur on the plantar aspect of the foot, adjacent to the metatarsal bones. They are caused by pressure necrosis from heavy body weight on wire cage floors. Lesions of various sizes consist of well-defined, ulcerated areas in the skin covered by a crusty scab. Secondary bacterial infections and abscesses may occur in the adjacent dermis. Microscopically, it consists typically of a circumscribed, ulcerated area covered by granulation tissue and necrotic debris with purulent exudate on the surface. The condition most commonly affects heavy, mature adults. Additional factors such as poor sanitation, trauma from wire-bottom cages with rough edges, and hereditary predisposition may contribute to the occurrence of the disease. *S. aureus* is frequently isolated from these lesions (Percy and Barthold, 2001). Incidences can be reduced by housing animals in solid-bottom cages with soft bedding material.

Moist dermatitis, also known as sore dewlap or hutch burn, is the result of constant wetting of the fur. It may be caused by panting associated with high environmental temperatures, drooling because of malocclusion; drinking from open pans; cold, damp contact bedding; or continuous wetting of the fur because of experimental design. The dermatitis that results is a chronic, progressive disease with various degrees of bacterial involvement. Inflammation in the subcutaneous tissues may progress to suppuration and abscesses with ulceration of the overlying skin. The most common bacterial agent is *Fusobacterium necrophorum* that causes necrobacillosis (Bergdall and Dysko, 1994). Another organism sometimes associated with moist dermatitis is *Pseudomonas aeruginosa* that can result in discoloration of the fur, blue fur disease (Richardson, 2000). Prevention consists of elimination or reduction of the causes to assure that the fur is kept clean and dry.

In addition to pododermatitis, dermatitis at other sites can be caused by bacterial infections. *S. aureus* is the most commonly identified pathogen. These infections vary in duration and range from a localized mild dermatitis with slight subcutaneous swellings to abscesses, septicemia, and death (Percy and Barthold, 2001). These subcutaneous abscesses usually contain a thick, tan or

white creamy exudate and may be surrounded by a fibrous capsule. *Corynebacterium pyogenes* and *P. multocida* also can cause suppurative and ulcerative skin lesions in rabbits. Streptococci have been reported to cause septicemic infections in young rabbits. Skin lesions affecting the genitalia, perineal region, nose, mouth, and eyelids can result from infections caused by *T. cuniculi*. These lesions may be raised, crusted, or ulcerated.

Multifocal aggregates of lymphocytes can be found surrounding hair follicles or in the deep dermal areas of untreated, healthy rabbits. The stimulus for their development is unknown.

In addition to the skin conditions described earlier, inflammation can also result from lacerations and abrasions caused by sharp objects in the environment, for example, nails and wire, as well as the teeth and claws of other rabbits when fighting. Painful skin problems can change the temperament of affected rabbits for the worse (Stein and Walshaw, 1996).

Viral infections of rabbits can cause neoplasia involving the skin and connective tissues. These viruses are not a problem in toxicology studies. For details about these virus infections, see DiGiacomo and Mare (1994).

Skin tumors other than viral fibromas or fibromatosis, myxomatosis, and papillomas include lipoma, squamous cell carcinoma, trichoepithelioma, basal cell tumors, and melanomas (Weisbroth, 1994).

Mammary Gland

Does have four or five pairs of mammary glands that extend in broad bands from the throat to the groin (Richardson, 2000). The milk is high in fat and protein (Harkness et al., 2010). Inflammation of the mammary glands, mastitis or "blue breast," is sometimes observed in recently kindled does. It is most prevalent in does that are heavy milk producers. Teat traumatic injuries including biting of the nipples and unsanitary conditions are additional predisposing factors. Mastitis is not limited to lactating does and may also be seen in pseudopregnant does that are maintained in unsanitary conditions (Richardson, 2000). In severe cases, the doe and young rabbits may die. Clinical signs typical of inflammation include fever, decreased appetite, abnormal milk, and infected swollen, firm mammary glands. The overlying skin has a red to dark-blue discoloration. On section, the affected gland may contain material having various appearances ranging from watery fluids to thick purulent exudates. Abscesses may form containing a thick, tan or white creamy exudate and be surrounded by a fibrous capsule. *Staphylococcus*, *Pasteurella*, and *Streptococcus* spp. are the most common causative agents.

Cystic mammary gland hyperplasia is associated with a condition termed cystic mastitis. This lesion occurs in one or more glands of nonbreeding does over the age of 3 years and appears to be a preneoplastic change. Often it is associated with uterine hyperplasia and uterine adenocarcinomas. Clinical signs are a nonpainful swelling of the affected gland and a bluish skin color with no evidence of inflammation. The nipple may have a brown serosanguinous discharge. Masses or fluid-filled cysts are sometimes palpable within the mammary tissues (Richardson, 2000). A biopsy can be useful in distinguishing between cystic hyperplasia and neoplasia.

Mammary gland dysplasia has been associated with pituitary adenomas and uterine adenocarcinomas. Benign and malignant tumors of the mammary gland are less common than uterine adenocarcinomas and most often occur in multiparous females that are 3–4 years of age. Papillomas and adenocarcinomas have been observed, especially in some families of breeding rabbits (Weisbroth, 1994). Animals with mammary adenocarcinomas frequently also have uterine adenocarcinomas.

Musculoskeletal System

Rabbits must be picked up and held correctly to prevent both animal and human injuries. Because they are very shy animals and are easily frightened, they often struggle and try to escape.

If a rabbit is picked up incorrectly or not held securely, it will kick violently with its hind legs, jump, or thrash (Suckow and Douglas, 1997). The rabbit skeleton is fragile, almost birdlike, and comprises only 7%–8% of the total body weight, whereas the skeletal muscle constitutes over 50% of the body weight (Percy and Barthold, 2001). Because the long bones and lumbar spine are surrounded by powerful muscle masses, the rabbit is particularly susceptible to fractures of the legs and back (Harkness et al., 2010). Traumatic lumbar vertebral fracture, usually in the lumbosacral region at L6 and L7, results in posterior paresis or paralysis following damage to the spinal cord. The onset is sudden, often after thrashing during restraint or handling without proper support of the hindquarters. Depending upon the location and severity of the fracture, the results are partial or complete motor paralysis with loss of control of the urinary bladder and anal sphincter. After a few weeks of intensive nursing care, function may return and the affected rabbit may show no obvious signs of a previous injury (Richardson, 2000). However, if the injury is serious enough, it may be necessary to euthanatize the animal. Young rabbits can fracture legs if there are holes in wire cages large enough to trap their feet. Geriatric rabbits can develop paresis and paralysis as a result of vertebral degeneration in association with aging. Vertebral spondylosis is common (Richardson, 2000).

Deficiencies in vitamin E can result in muscle damage. Affected rabbits have muscle soreness and stiffness. At necropsy, gross findings consist of pale mineralized streaks in skeletal and cardiac muscle. Common gross sites are the diaphragm, paravertebral regions, and the hind limbs. Microscopic findings are hyaline degeneration and necrosis of affected myofibers with mineralization of the sarcoplasm and histiocytic or granulomatous inflammation. Later lesions have collapse of the sarcolemmal sheaths and interstitial fibrosis (Percy and Barthold, 2001).

Rabbits, especially adults, are very sensitive to vitamin D toxicity. This toxicity results in calcification of many organs and tissues. Increased resorption of calcium from bone leads to osteodystrophy with osteoid dysplasia and osteosclerosis. Microscopically, the bone lesions are characterized by excessive production and deposition of a highly cellular, abnormal basophilic osteoid with many active osteoblasts. These changes are observed on the periosteal and endosteal surfaces, medullary trabeculae, and haversian systems (Percy and Barthold, 2001).

Young rabbits with "splay leg" keep one or more legs spread and lack the ability to adduct the affected limbs. They cannot come to a standing position. These rabbits can have a variety of congenital abnormalities including inherited syringomyelia, hypoplasia pelvis, femoral luxation, and distal foreleg curvature. Greene (1965) investigated several hereditary disorders in rabbits, including oxycephaly, brachydactylia, dwarfism, cretinism, dwarf-cretin complexes, and hydrocephalus. Also he studied the role of heredity in the toxemia of pregnancy and neoplasia. Large doses of vitamin A given to pregnant rabbits can produce a high incidence of gross congenital malformations (Shenefelt, 1972).

The mandible of rabbits is the most frequent site for inflammation of the bone, osteomyelitis, and rare bone neoplasms, that is, osteosarcomas (Stein and Walshaw, 1996). An osteoma (Stedham, 1976), osteochondroma, and osteosarcoma involving the appendicular skeleton (Weisbroth, 1994) have been reported.

Digestive System

Rabbits are herbivores with a large and relatively complex digestive tract that fills a capacious abdominal cavity. Sometimes, they have been referred to as pseudoruminants (Arrington, 1978). The stomach typically contains approximately 15% of the ingesta present in the digestive tract. The small intestine is relatively short for a mammal having approximately 12% of the total volume of the gastrointestinal tract. Rabbits are hindgut fermenters. Based on the body weight, the rabbit has the largest cecum of any living mammal. The cecum typically holds about 40% of the

ingesta present in the digestive tract. Fine particulate materials are selectively channeled into the cecum during the passage through the large intestine, while larger particulate material composed of undigestable fiber is usually directed into the colon and passed as dry fecal pellets. At night, rabbits practice coprophagy, that is, they consume small, mucous-coated soft moist fecal pellets; cecotropes, high in B vitamins; and protein produced during normal cecal fermentation. Lymphoid tissues are abundant in the intestinal tract of rabbits. Lymphoid (Peyer's) patches are large and prominent in the small intestine; however, most of the lymphoid tissues are found in the large intestine. The gut-associated lymphoid tissue (GALT) represents over 50% of the total mass of lymphoid tissue in the body, which may account for the relatively small spleen seen in rabbits. A common incidental finding in rabbits is the presence of large histiocytes filled with particulate debris in the follicular centers of GALT.

Not only is it important to understand how different parts of the digestive tract function in the rabbit, but it is also important to understand the importance of a proper diet. A balanced diet with adequate amounts of plant fiber is necessary to ensure normal gut motility and thereby prevent or control several diseases of the digestive system (Richardson, 2000).

Normally, the stomach will effectively sterilize the food consumed with its pH of 1–2. Nutrients are absorbed in the small intestine from the fluid ingesta exiting the stomach. In the large intestine, as noted earlier, the indigestible fiber is processed into hard fecal pellets and the smaller particles are fermented in the cecum before being processed into cecotropes. When conditions for movement of ingesta are disrupted, disease can follow. The transit time for food in the rabbit digestive tract is normally short. Diets high in indigestible fiber, especially lignocellulose, stimulate the rapid movement of ingesta through the gastrointestinal tract. Diets low in fiber lead to reduced gut motility and gastric stasis with increased incidences of impaction. Diets with high sugar and starch contents exceed the small intestine's ability to absorb them and their passage into the cecum where proliferation of microorganisms can result in enterotoxemia. Anorexia and other illnesses may cause the pH of the stomach to rise reducing the effectiveness of the stomach to sterilize the contents. Also, a compact ball of concentrated dry food in the stomach can prevent effective sterilization allowing microorganisms to pass further down and cause adverse effects (Richardson, 2000). If the ball of food becomes large enough, impaction will result.

A high-fiber, low-protein, and low-carbohydrate diet is more natural for the rabbit. The digestive system of the rabbit works best when provided ample plant fiber such as diets containing good-quality hay with decreased amounts of pelleted diets. Also, offering leafy greens is advisable. Changes in the diet such as new vegetables should be done gradually. Starch and fat should be minimal. Proper diets will help prevent many problems of the digestive tract. Rabbits need to be able to exercise daily.

Teeth

The dental formula for a rabbit's 26–28 teeth is 2 (2/1 incisors, 0/0 canines, 3/2 premolars, and 2–3/3 molars). Rabbits have a set of small incisors called peg teeth directly behind the front incisors. These small incisors are thought to protect the upper gum during chewing (Richardson, 2000). The upper incisors fit anteriorly to the bottom incisors. Folds of skin between the incisors and premolars form an antechamber with limited visibility and access to the rest of the oral cavity (Cruise and Brewer, 1994).

Malocclusion is not life threatening but could have an adverse effect on a research study. All rabbit teeth have open roots and therefore grow continuously. Incisor teeth of rabbits can grow 10–12 cm a year (Harkness et al., 2010). Malocclusions occur when the incisors do not properly occlude and, therefore, are not worn away. The most common cause is an inherited shortening of the upper jaw, mandibular prognathia, or the result of fractured or missing teeth following head injuries.

Affected rabbits have weight loss, difficulty in chewing their food, and excessive drooling, or ptyalism (Suckow and Douglas, 1997). This condition can be easily managed by clipping the teeth on a regular basis.

Ulceration of the mouth is common. The first signs are salivation and anorexia. The cause is malocclusion of the cheek teeth. The lower molars cause ulcerations on the tongue, whereas the upper molars generally grow laterally and cause buccal ulcerations of the cheek (Richardson, 2000).

Tongue

The tongue is large and covered by spine-like structures called filiform papillae that have no taste buds. The base of the tongue has a pair of large circumvallate papillae on the dorsal surface, which is occasionally mistaken for papillomatous growths. Also, rabbits have rows of well-developed foliate papillae on the caudal, lateral border and fungiform papillae on the rostral border of the tongue. The circumvallate, foliate, and fungiform papillae are all shown to have taste buds (McLaughlin and Chiasson, 1979).

Oral papillomatosis (warts) is a rare virus disease in rabbits. It results in white lesions on the underside of the tongue that may become ulcerated. The lesions are limited and usually regress after several weeks (Richardson, 2000).

Salivary Glands

Four pairs of salivary glands are present, the parotid, submaxillary, sublingual, and zygomatic. The largest are the whitish-brown parotids. They extend below and in front of the base of the ears. Submaxillary are oval glands at the angle of the mandible. Sublingual are small, flattened glands adjacent to the mandible and the submaxillary glands. The zygomatic, also termed the infraorbital, glands lie in the anteroventral angles of the orbit, just below the lacrimal glands.

Stomach

The stomach has cardiac, fundic, and pyloric portions. The cardiac portion is thin walled and relatively immobile, with numerous small glands. The exocrine secretory region of the highly glandular fundus is the major source of acid for the pH of 1–2 in the stomach. It has gastric pits lined with parietal (oxyntic) cells that secrete acid and intrinsic factor and peptic (chief) cells that secrete pepsinogen. The pyloric region has thick layers of smooth muscle. Submucosal areas often contain heterophils and lymphocytes. Grossly, the mucosa often appears to have red foci that are not apparent microscopically. These areas are assumed to be areas of hyperemia that bleach out during fixation. Because of its thin wall, the stomach must be handled with care. It is easily ruptured during necropsy. Also, the stomach undergoes autolysis rapidly after death and may spontaneously rupture before the necropsy can begin (Feldman and Seely, 1988).

Fastidious grooming habits predispose rabbits to ingestion and subsequent accumulation of hair leading to the formation of hair balls (trichobezoars) in the stomach or pylorus. The rabbit is unable to vomit because of a well-developed cardia sphincter, so these accumulations cannot be removed by that route. Hair balls are common in the stomach, especially in older adult animals that lick or pull their own fur. Reduced gastric motility from low-fiber diets, anorexia, a lack of exercise, and long haired breeds are predisposing factors for the development of hair balls (Richardson, 2000). They generally do not cause any problems and are only noted incidentally at necropsy. In a few rabbits, the hair mass becomes large enough to obstruct the flow of food with anorexia, weight loss, and decreased fecal output. If the obstruction is prolonged, death may result. Gastric rupture with peritonitis has been observed (Suckow and Douglas, 1997). Hair balls are less common in the duodenum.

Small Intestines

The small intestines consist of a relatively long duodenum with a short jejunum and ileum. Brunner's glands are distributed throughout the length of the duodenum. The common bile duct from the liver and gallbladder opens into the duodenum near its origin at the pylorus, and the pancreatic duct opens into the duodenum near the origin of the jejunum. The wall of the jejunum is thinner and appears less vascularized than the duodenum. Lymphoid aggregates (Peyer's patches) are large and prominent throughout the jejunum and ileum. A rounded enlargement, the sacculus rotundus, is formed where the ileum ends at the ileocecal valve.

Marked plasma cell infiltrations in the intestinal tract have been described as "intestinal plasmacytosis" in rabbits used in research studies. Older rabbits were particularly at risk, especially those used for antibody production or cholesterol studies. The lesions were frequently only observed during microscopic examinations of the tissues. Severely affected animals had grossly thickened intestinal mucosa. Microscopically, lesions were most prominent in the small intestine and cecum with less involvement of the lymphoid tissues of the sacculus rotundus or cecal appendix. Variable degrees of mucosal erosion, dilatation of lacteals, and blunting of the overlying villi occurred in the small intestine. Marked infiltrations of well-differentiated plasma cells with uniform sizes and shapes were observed in affected tissues. When severe, infiltrations of dense aggregates in the lamina propria completely replaced the normal architecture. These infiltrates were usually confined to the intestinal tract; however, in a few animals, plasma cells were increased in the spleen and mesenteric lymph nodes.

Intussusceptions are uncommon, but usually occur in young rabbits with enteritis. Anorexia and a cessation of droppings are clinical signs in affected rabbits.

Large Intestines

The large intestines consist of a spiral cecum, a sacculated colon, and the rectum. At the ileocecal junction adjacent to the sacculus rotundus, the cecum has a thickened round patch of lymphoid tissue called the cecal tonsil. The cecum is very large with thin and smooth walls. The inner surface is greatly increased by a long spiral fold or valve. The cecum terminates in the vermiform or cecal appendix, a thick-walled narrow blind tube with abundant lymphoid tissues. The grossly thickened walls of the sacculus rotundus, cecal tonsil, and vermiform appendix are due to aggregates of organized lymphoid tissue and macrophages in the lamina propria and submucosa. The colon can be divided into three major segments: the ascending, transverse, and descending colons. These segments are characterized by numerous pouches or haustra formed between bands of tissue termed taeniae. In the ascending colon, the taeniae form three rows of haustra. The haustra gradually decrease to a single row in the descending colon. The ascending colon is a long and coiled tube that ascends from the ileocecal junction to the liver. Because the ascending colon is within the pelvic cavity, this location can be used to collect accurate sections of the colon for microscopic examination. The transverse colon is a short segment between the ascending and descending colons. Along the greater curvature of the junction of the transverse and descending colons is a slightly curved spindle-shaped structure, the fusus coli that is unique to lagomorphs. The fusus coli is an area 5–8 cm in length of thickened circular muscle and mucosa that has numerous ganglion cell aggregates. The mucosa here is 4–5 times thicker than in the remainder of the descending colon. The fusus coli serves as a pacemaker regulating the movement of ingesta into the descending colon and transporting more fluid chyme back into the cecum (Cruise and Brewer, 1994). The long descending colon terminates in the short, straight rectum and anus. Lymphoid patches can be found throughout the submucosa of the cecum and colon. Sections of the rectum often contain submucosal lymphocytes that do not form aggregates or foci.

In the cecum, bacteria ferment small pieces of cellulose to produce B vitamins and volatile fatty acids that are contained in cecotropes. The predominant organisms are *Bacteroides* spp. with smaller numbers of *Clostridium* sp., *E. coli*, and *Streptococcus faecalis*. Saccharomycetaceae yeasts are considered to be part of the normal flora and nonpathogenic. The *Bacteroides* are Gram-negative cellulolytic anaerobes. The cecotropes have a mucilaginous coating that forms a capsule to survive passage through the stomach's acidity after ingestion. Nutrients from the cecotropes are absorbed in the small intestine (Richardson, 2000). When excessive fermentable carbohydrates are fed, the cecal contents become more acid. The amount of normal flora is decreased and the *Clostridium* sp. proliferate. The result is enteritis. As the fiber content of the diet goes down, numbers of *Clostridium* sp. and *E. coli* increase. Reduced intestine motility is another result of a low-fiber diet. Because the stomach and cecum empty very slowly, the rabbit produces fewer and fewer fecal pellets. The rabbit will stop eating. This may be followed by impaction from a mass of ingesta. Feeding of diets that are high in protein, low in fiber, and high in carbohydrate or sugar will cause rabbits to produce more cecotropes than are needed with accumulation of excess cecotropes on the fur. Other causes for the accumulation of excess cecotropes include rabbits that are overfed who lose the urge for coprophagy, large rabbits in hutches so small that they do not have room for coprophagy, and rabbits that are very overweight and have excessive dewlap, incisor or molar malocclusion, or arthritis and spinal pain preventing coprophagy (Richardson, 2000).

Diets low in fiber result in reduced motility and the buildup of food material in the cecum. This excess in food material can result in a cecal impaction. Fermentation of the excess food material can lead to tympany. Further, the presence of excessive amounts of sugars or starch can result in an altered pH of the cecum contents with proliferation of *Clostridium spiroforme* and a fatal enterotoxemia.

Young rabbits experience extensive stress during shipment and upon arrival a testing or research facility including variations in diet and temperature as well as unusual noise and unfamiliar handling. This stress can predispose these young animals to infections by agents in the new environment and agents that may have been carried from the production facility.

"Enteritis complex" has been used to encompass the multifactorial group of enteric diseases of rabbits, especially those 5–12 weeks of age. The most common clinical sign is diarrhea. It is associated with many causes including dietary changes and bacterial, protozoal, and viral infections, following antibiotic use or at times of stress. A combination of several causative factors is often involved. The higher stomach pH (3–7) in rabbits with diarrhea allows bacteria to pass into the large intestine. Several causative factors have been shown to be associated with enteritis including the stress of weaning, changes in diet, insufficient fiber, carbohydrate overload with subsequent alterations in gut flora and intestinal pH changes, crowding, and transport. Infectious agents are now recognized to have an important role in causing enteritis. These agents include rotaviruses, coronaviruses, Clostridia, *E. coli*, *Lawsonia*, *Salmonella*, *Vibrio*, and coccidia. Mucoid enteropathy does not appear to be caused by a specific infectious agent, but may be associated with bacterial toxins, although the pathogenesis is unclear. A condition known as antibiotic toxicity occurs when certain antibiotic agents alter the normal intestinal flora allowing an overgrowth by enterotoxic Clostridia. Enteric diseases vary greatly in their outcome. Some are transient with little effect on the health of the rabbit, whereas others are often fatal. Some specific disease entities are discussed below and in more detail in Percy and Barthold (2001) and Manning et al. (1994).

Enteritis in newborn or suckling rabbits may be precipitated by hypothermia and maternal neglect. The most common bacterial organisms associated with enteritis in newborn or suckling rabbits are enteropathogenic strains of *E. coli* and *Staphylococcus* spp. (Percy and Barthold, 2001). The clinical signs are usually a yellow watery diarrhea that stains the perineum and abdomen. Within a litter, the morbidity and mortality may reach 100%. At necropsy, the gross findings are milk in the stomach, whereas the remaining digestive tract is filled with watery contents. Isolation of *E. coli* is significant in rabbits under 2 weeks of age because it is not normally present in the flora at this age. Many of these young rabbits die as a result of septicemic infections.

Weanling rabbits of 4–8 weeks of age are more susceptible to enteric diseases than adults. Up to 21 days, protective antimicrobial fatty acids found in the stomach of newborn and suckling rabbits control the digestive tract microflora. After 21 days, these protective fatty acids decrease and the large intestine acquires its bacterial flora. At the time of these changes in the digestive tract, these rabbits become more susceptible to enteritis (Richardson, 2000). Other causative factors include the stress of weaning, changes in diet, insufficient fiber, carbohydrate overload with subsequent alterations in gut flora and intestinal pH changes, crowding, and transport. The clinical signs in these young rabbits are profuse diarrhea, dehydration, and a rough hair coat. Mortality can reach 100%. Lesions occur in the cecum that resemble those of *C. spiroforme* enterotoxemia. The predominant findings are hemorrhage and edema of the cecum with watery or mucoid blood-tinged contents.

Several species of *Clostridium* have been implicated in enteritis, and enterotoxemia in rabbits including *C. perfringens*, *C. difficile*, and *C. spiroforme*. *C. piliforme*, the cause of Tyzzer's disease, will be discussed separately. These organisms are Gram-positive bacilli that reside in the large intestine and grow under anaerobic conditions. After significant changes in the diet, pathogenic strains are able to produce powerful enterotoxins or cytotoxins that can result in severe and often fatal enteric disease. Early studies demonstrated the type E "iota" toxin in fatal cases of enterotoxemia that was believed to have been produced by *C. perfringens*. Other studies have demonstrated that the type E "iota" toxin can be produced by *C. spiroforme*. Fatal colitis and enterotoxemia have been associated with overgrowth of *C. difficile* following prolonged treatment with antibiotics against Gram-positive organisms such as procaine penicillin, cephalexin, erythromycin, ampicillin, amoxicillin, clindamycin, lincomycin, tylosin, and metronidazole. The toxic effects of these antibiotics vary from animal to animal. *C. difficile* infection was observed in specific pathogen-free rabbits in the absence of antibiotic treatment. The organism was found in the small intestine and cecum. It was able to produce toxins A and B (Percy and Barthold, 2001).

Enterotoxemia generally occurs within the first 24–96 hours after shipment during the quarantine period and is rarely, if ever, diagnosed in animals after they have been removed from the quarantine regimen. Clinically, these animals appear to be somewhat depressed, inactive, and anorexic and often have a greenish brown discoloration on the perineal area. The diarrhea is moderate to severe. These animals are generally dehydrated, but death often occurs before dehydration is apparent. Affected animals often have a high death rate.

Gross findings depend upon the course of the disease. In acute illness, the carcass is usually in good condition with perineal staining of the fur. In subacute to chronic cases, carcasses are usually thin and dehydrated. There is extensive staining of the perineum, belly, and rear legs with watery green to tarry brown feces. Straw-colored fluid may be present in the peritoneal cavity. Extensive ecchymotic hemorrhages are usually present in the serosal of the cecum, sometimes with the distal ileum and proximal colon affected. Ecchymoses can also occur in the epicardium and thymus. The large intestine, especially the cecum, is frequently dilated with watery to mucoid, green to dark-brown contents with gas formation. The affected large intestine can be markedly thickened as a result of edema. The mucosa findings can vary from hemorrhage to ulceration and fibrinous exudates. Microscopic changes in the cecum are those of a necrotizing typhlitis with irregular denuding of the mucosa, ulceration, fibrinous exudation, and leukocytic infiltration, predominantly heterophils. The mucosal epithelial cells, enterocytes, can be swollen, vacuolated, flattened, and denuded or undergo replacement proliferation. There can be relative sparing of the crypt bases and the lamina propria. The mucosa and submucosa are usually congested, with frequent hemorrhages and thrombi. Large numbers of Gram-positive bacilli can often be demonstrated adhered to the mucosal surface. In animals that survive the earlier necrotic changes, the mucosa undergoes repair characterized by epithelial hyperplasia. At this stage, the clinical signs are those of malabsorption with continued diarrhea (Percy and Barthold, 2001).

Another major source of enteritis in commercial rabbitries and occasionally in research facilities is colibacillosis, caused by enteropathogenic strains of *E. coli*. Colibacillosis results in an acute,

sometimes explosive diarrheal disease that often produces a high mortality rate in young rabbits in the 10–20-week age group. *E. coli* is normally absent or present in small numbers in the digestive tract of suckling and weanling rabbits. The absence or small numbers are attributed to the low pH of the stomach. Therefore, the stomach and small intestine are usually relatively free of these bacteria. Colibacillosis is an infrequent problem in laboratories that provide good care and adequate diet. Clinical disease is associated with rapid proliferation of *E. coli* and a shift from the normal predominantly Gram-positive in the large intestine to a Gram-negative bacterial flora. This rapid growth can be associated with several factors, including abrupt changes in diet or management, experimental studies with compounds such as antibiotics that may affect the bacterial flora of the intestinal tract, intestinal coccidiosis with its accompanying mucosal damage and resorption of endotoxins, and a change in cecal pH. The normal pH of the large intestine exerts an antibacterial effect in rabbits. Strains of *E. coli* isolated during outbreaks of this disease are classified as enteropathic. These strains cause intestinal disease, do not produce enterotoxins, and are not considered to be enteroinvasive. Isolates vary widely in pathogenicity as well as their response to antibiotics and improved sanitation. The ability of these isolates of *E. coli* to attach to mucosal epithelial cells of the small or large intestine is closely related to the age of the rabbits from which they were isolated (Percy and Barthold, 2001). Although *E. coli* is often the predominant bacteria isolated, it should be noted that this organism also can be secondary to the primary cause of death.

Gross findings are dehydration and a perineal region stained with watery yellow to brown feces. The small intestine usually appears normal. Frequently, the cecum and colon are distended with watery yellow to gray-brown contents. Other findings are edema with mucosal and subserosal ecchymotic hemorrhages in the cecum or colon, edematous mesenteric lymph nodes, and prominent lymphoid tissues in Peyer's patches and the sacculus rotundus. Microscopic changes in suckling rabbits with coliform enteritis are characterized by large numbers of coccobacilli attached to the mucosal epithelial cells in both the large and small intestines with infiltration of the lamina propria by heterophils. Microscopic lesions in weanlings are usually more extensive with blunting of the villi in the ileum, edema of the lamina propria, and leukocytic infiltration, predominantly by heterophils. Epithelial cells at the tips of the villi in the small intestine and at the tips of the large intestine crypts are swollen and have attached bacterial colonies. Also, the cecum and colon frequently have detachment and ulceration of the epithelium at the tips of the cecal folds.

Colibacillosis occurs less frequently in rabbits at any age past weaning. The disease can range from a mild diarrhea and weight loss to a severe diarrhea with mortalities up to 50%. The diarrhea can lead to intussusception or rectal prolapse. After recovery, affected rabbits may remain undersized. Gross examinations can reveal in "paintbrush" hemorrhages on the cecum and colon serosal.

Another form of bacterial enteritis characterized by a sudden outbreak of profuse watery diarrhea, stained hindquarters, and dehydration is a component of Tyzzer's disease caused by *C. piliforme*, previously named *B. piliformis*. This disease clinically resembles mucoid enteritis and has a short course of illness with mortality rates of 10%–50%, usually affecting young rabbits, 10–12 weeks of age, but can occur in adults and preweanlings. It is transmitted through ingestion of feces of infected animals. The onset of illness is often associated with stress such as overcrowding, excessive heat, or experimental procedures that allow the organisms to multiply (Percy and Barthold, 2001). Gross findings are usually extensive ecchymotic hemorrhages, occasionally with fibrinous exudate on serosal surfaces in the cecum and colon. Frequently, the liver has disseminated pale foci. Less frequently, the myocardium has pale, linear streaks, particularly near the apex of the left ventricle. Animals that survive the initial disease have thin carcasses and usually identifiable circumferential regions of fibrosis and stenosis in the terminal ileum or large intestine. Microscopic changes are consistently present in the intestinal tract, usually in the liver, and infrequently in the myocardium. The intestinal tract has focal to segmental necrosis of the mucosa of the cecum with variable involvement of the distal ileum and proximal colon. Large numbers of bacteria are often

present on the damaged mucosal surface. The affected intestine wall has extensive edema, muscle necrosis, and leukocytic infiltrates, predominantly heterophils. Liver lesions are characterized by periportal foci of coagulation or caseation necrosis often with a conspicuous scarcity of peripheral inflammatory cells. Long filamentous intracytoplasmic organisms in characteristic bundles stained by the Warthin–Starry silver or Giemsa methods can often be found in or near the areas of hepatic necrosis. Tyzzer's bacilli are not present in lesions examined after the acute phase of the disease. Heart lesions consist of focal or linear areas of coagulation necrosis with a minimal inflammatory response (Percy and Barthold, 2001).

Mucoid enteritis or enteropathy is one of the most common diseases affecting weaned young rabbits 7–14 weeks of ages, a vulnerable time when the cecal microflora is still becoming established. It does not appear to be caused by a specific infectious agent but is associated with changes in the diet that decrease the cecal pH and disrupt the normal cecal flora. A possible role for bacterial toxins has been suggested. Inadequate dietary fiber with cecal and colonic impactions and use of antibiotics such as clindamycin and lincomycin appear to contribute to this disease. Mucoid enteritis rarely occurs in rabbits that are fed a high-fiber diet with limited amounts of grains, proteins, and fats. Enteritis associated with extensive mucus production in older rabbits is usually a part of the "enteritis complex" and has a lower mortality rate. Affected rabbits produce abundant gelatinous or mucoid feces. Other clinical signs include mucoid diarrhea, depressed food consumption, depression, a bloated abdomen, grinding of the teeth, hypothermia, weight loss, polydipsia, hunched posture, and the perineal region stained with yellow- to brown-colored fecal material. Mortalities are very common in these young rabbits. Up to 60% of affected rabbits may have an accompanying pneumonia. Surviving rabbits grow very slowly. Gross findings include a stomach and sections of the intestines filled with gas or a watery fluid, excessive production of mucus in the cecum, gelatinous contents in the colon, and cecal or colon impaction without evidence of mucosal inflammation. A characteristic microscopic finding is marked noninflammatory goblet cell hyperplasia in the small and large intestine. This finding is consistent with the clinical observations (Percy and Barthold, 2001).

Salmonellosis is an uncommon enteric disease in rabbits that is usually associated with contaminated food or water. The most frequent types isolated are *Salmonella typhimurium* and *S. enteritidis*. Clinical signs include septicemia, depression, pyrexia, and rapid death, often accompanied by diarrhea. Pregnant does may abort. Gross findings of septicemia are diffuse petechial hemorrhages and vascular congestion of various organs. Does can develop metritis. Necrotic pale foci are found on the liver and spleen. Lymph nodes are edematous (Percy and Barthold, 2001).

Infections by *Lawsonia intracellularis*, previously named *Campylobacter*-like organism, can result in proliferative and histiocytic lesions in the small intestines of rabbits as well as a number of species including rats, hamsters, and guinea pigs. This is an obligate intracellular bacterium that can reach large numbers within the enterocytes of the small intestine. Infections are common in some rabbit colonies. Lesions are often mild and found as incidental findings in affected rabbits. Clinical signs vary with the stage of infection and the age of the affected rabbit. Acute infections can result in diarrhea and death in suckling, weanling, and young adult rabbits. Gross findings in the acute disease may consist of semifluid mucinous contents in the colon and rectum. In more chronic infections, there may be thick opaque loops of small intestine. In these animals, the mucosal surface of the small intestine has a markedly thickened and rugose appearance (Percy and Barthold, 2001). Microscopic findings in the mucosa vary from acute inflammation with erosions and suppuration to proliferation with multifocal to diffuse hyperplasia of enterocytes lining crypts and villi associated with a prominent histiocytic infiltration accompanied by occasional multinucleated giant cells in the lamina propria. Special stains reveal typical clusters of small bacteria in the apical cytoplasm of enterocytes (Percy and Barthold, 2001).

Other bacteria that have been associated with enteritis and diarrhea include *Vibrio* sp. and *Pseudomonas* sp. infections from contamination of drinking water.

Coccidiosis is the most common parasitic disease in rabbits. Twelve species of protozoan organisms are known to infect the rabbit, all in the genus *Eimeria*. The disease has two forms: one affecting the liver and the other affecting the intestine. Transmission is by ingestion of sporulated oocysts that are in the feces. Cecotropes do not contain sporulated oocysts because cecotrophy does not allow time for sporulation. Therefore, cecotropes are not infective (Richardson, 2000).

Liver infections are caused by a single species *E. stiedae*. Clinical signs such as diarrhea and weight loss are often seen only in young animals; adults may show no physical change. This species colonizes the bile duct epithelium and causes an enlarged or irregularly shaped liver (Pakes and Gerrity, 1994). Affected livers may result in distended or pendulous abdomens. Grossly, numerous yellow or white spots may be present on and in the liver. Heavily infected rabbits may develop signs of bile duct obstruction with liver enlargement, ascites, jaundice, diarrhea, anorexia, and result in death (Richardson, 2000).

Intestinal coccidiosis can result from infections by several species, including *E. irresidua*, *E. magna*, *E. media*, and *E. perforans*. Although all of these species colonize intestines, the site and pathogenicity depend upon the specific species. *E. magna* has high infectivity for the small intestine, whereas *E. perforans* has low infectivity for the small intestine. *E. media* has moderate infectivity for both the small and large intestines (Richardson, 2000). Mixed infections with more than one species are common. Clinical signs vary depending on the extent of infection and the age of the animal. Young rabbits are most commonly affected having diarrhea, weight loss, and anorexia. The diarrhea frequently is characterized by blood and mucus. Severe diarrhea can result in intussusception. In mild infections, no clinical signs may be evident. Severe infections can result in dehydration and death due to secondary bacterial infections (Richardson, 2000).

At least two viruses are known to cause enteritis in suckling and weanling rabbits. Both rotavirus and coronavirus have been isolated. Both viruses affect rabbits between 3 and 12 weeks of age. Infections with rotavirus alone are often subclinical and can be endemic in some rabbit populations. In young rabbits under stress and exposed to other secondary agents, both rotavirus and coronavirus infections can result in severe clinical signs including anorexia, diarrhea, dehydration, and death. Adult rabbits can have subclinical coronavirus infections. The gross findings for both diseases are similar with intestines distended and congested as well as petechial hemorrhages in the small and large intestines. Microscopically, both viruses cause villous atrophy of the jejunum and ileum. Identification of which virus is involved requires virus isolation procedures (Richardson, 2000). A highly contagious enterocolitis has been reported in suckling, weanling, and lactating rabbits in Europe that appears to be caused by as yet unidentified virus. Clinical signs are characterized by anorexia, moderate watery diarrhea, reduced gastrointestinal motility that progresses to ileus, and death in 2–3 days. Gross findings are abundant fluid in the small intestine with gas and mucus in the colon (Richardson, 2000).

The nematode *Passalurus ambiguus*, a pinworm, is a common parasite of domestic rabbits. Adult worms are very small and found in large intestine, primarily in the cecum and colon. Juvenile stages are found in the mucosa of the small intestine and cecum. Infestations of small to moderate numbers are considered nonpathogenic with no clinical signs. Heavy infestations are reported to cause impaired weight gains, poor breeding performance, and occasionally death. Tapeworms are rarely seen in laboratory rabbits. More detailed information on parasites of rabbits is available in Pakes and Gerrity (1994), Hofing and Kraus (1994), and Percy and Barthold (2001).

Liver

The liver has two major lobes and several smaller ones. A deep median fissure at the falciform ligament divides the liver into a large right and smaller left lobe. The right lobe is further divided into a right medial lobe adjacent to the gallbladder, the quadrate lobe, and the small caudate lobe often found in the groove for the vena cava. The primary nonspecific microscopic changes consist of lymphocyte

infiltration in the periportal area and varying degrees of hepatocytic cytoplasmic vacuolation or fatty change. The incidence of lymphoid infiltration varies with age and is more common in females. The severity is generally minimal to mild. Vacuolation of hepatocytes is nonspecific but appears to be more common in females (Wells et al., 1988) and is greatly affected by ad libitum feeding of standard diets and fasting prior to blood collection (Weisbrode et al., 1990). Extreme fatty vacuolation of hepatocytes and hepatic necrosis can develop in pregnant, pseudopregnant, and postparturient females resulting in a condition known as pregnancy toxemia. A similar toxemia occurs in obese male rabbits. Although the cause of this disease appears complex, however, there are some consistent findings. The affected animals are obese and quit eating. Lactation may be an additional causative factor. The rabbits show signs of depression and anorexia and may abort or even convulse and die with few clinical signs of illness. The urine is clear. Urinalysis demonstrates evidence of protein and ketones. Gross findings are an empty stomach, obesity, a pale-yellow liver and kidneys, possible dead fetuses, and uterine hemorrhage. Hill et al. (1988) have described a spontaneous storage-like disease characterized by hepatocytes with expanded, foamy cytoplasm and distinct cell borders. These lesions occurred singularly or as multifocal aggregates. Necrosis of individual or small groups of hepatocytes with no zonal distribution is not uncommon. It is assumed that the etiology is circulatory in origin.

Cytoplasmic vacuolation of hepatocytes associated with glycogen accumulation is a variable finding seen microscopically in rabbits fed commercial diets (Percy and Barthold, 2001).

In rabbits, torsion of the caudate process generally results in a fatal subcapsular hepatic rupture several centimeters long with hemorrhage into the peritoneal cavity. The gross lesion resembles a hemorrhagic tumor. Microscopic examination reveals the lesion is an infarct (Ruebner et al., 1965).

A systemic viral disease has been reported in rabbits that results in hepatic necrosis and hemorrhages in the lungs, kidneys, and other tissues. (See the section "Cardiovascular System" for more details.)

Bile duct dilation with striking hyperplasia of the epithelium can be a feature of hepatic coccidiosis. Milder coccidial infections that heal can resemble in biliary cirrhosis (Ruebner et al., 1965). Bile duct tumors are relatively common spontaneous neoplasms in rabbits (Weisbroth, 1994).

Gallbladder

The gallbladder is located in a rounded cavity in the medial surface of the right medial lobe of the liver and contains a dark-greenish fluid. At necropsy, the gallbladder often has ecchymotic serosal hemorrhages. These hemorrhages rarely extend the full thickness of the wall. Mucosal hyperplasia with frond-like papillary projections has been observed in animals that were being fed 40%–50% of a normal libitum diet. It is assumed that this hyperplasia is in response to cholestasis or gallbladder distention.

Pancreas

The pancreas is a diffused irregular mass of tissues located in the mesentery of the small intestine. It often contains foci of lymphocyte infiltration within acinar areas with no apparent acinar necrosis. Individual acinar necrosis or granular depletion of the cytoplasm can be found in animals that have undergone prior fasting for 12–16 hours. The islets are microscopically similar to other species. New Zealand White rabbits have been reported to develop a disease resembling maturity-onset diabetes mellitus.

Respiratory System

Rabbits are very sensitive to heat. Clinical signs of heat prostration include rapid respiration, cyanosis, and prostration. Does near kindling and young rabbits in nesting boxes are particularly

prone to temperature extremes. Open mouth breathing is an indication of severe respiratory distress and carries a guarded prognosis (Richardson, 2000). Normally, rabbits are obligate nose breathers. Twitching of the nostrils is normal behavior in active rabbits; however, it may be absent in resting or sick. Nasal and ocular discharge, anorexia, labored breathing, abscesses, reduced reproductive ability, head tilt, and circling are usually associated with *P. multocida* infections. Some breeds of rabbits, such as the Flemish Giants, appear to be more susceptible to *P. multocida* infections than others.

Nasal Cavity

The nasal cavity presents no unusual anatomical structures. The respiratory surface may contain multifocal areas of mucoid material. The submucosa may contain individual or small aggregates of lymphocytes, but no lymphoid follicles have been recognized.

Snuffles is a rhinitis or sinusitis characterized clinically by a mucous or mucopurulent nasal discharge that stimulates the animal to rub the nares with the medial surface of the front limbs and paws. The resulting wetness of the paws may be visible even though the nasal area exudation is dry. Sneezing, coughing, and respiratory "snuffling" are common symptoms. The gross and microscopic lesions are usually limited to the nasal cavity. Purulent exudate is present on the surface of the cavity or turbinates with heterophils (neutrophils) and edema present within the submucosa or on the mucosal surface. Gram-negative rod-shaped bacteria may be found within the exudate with the aid of special stains. Usually, *P. multocida* is isolated from the exudates. The incidence varies from 20% to 80%, but with the establishment of breeding colonies free of *P. multocida*, the current incidence may well be below 10%. Rabbits commonly are infected with this organism without any clinical signs. After being stressed, these rabbits can begin sneezing and coughing, followed by a nasal discharge. The snuffles may persist, spread to the middle ears, or progress to pneumonia and septicemia.

Trachea and Lung

The trachea is an unpaired tubular air passageway that extends from the larynx to where it divides into primary bronchi. It is supported by segmented incomplete cartilage rings and lined by respiratory epithelium with histologic features similar to those of rats and mice. The lungs are divided into six primary lobes with both the left and right lungs having apical, cardiac, and diaphragmatic lobes. The left apical lobe is very small. The right diaphragmatic lobe is further divided into a large lateral and a smaller accessory lobe (Wingerd, 1985). There are peribronchiolar lymphoid foci that tend to increase in size with age. There are no respiratory bronchioles in the rabbit.

Several of the barbital-based anesthetic agents cause grossly apparent petechial hemorrhages on the surface of the lungs that disappear with fixation. Macrophage-like cells with foamy-appearing cytoplasm can be found within the alveolar spaces of untreated, control rabbits. These cells can occur in lungs that have no other evidence of inflammation.

Enzootic pneumonia is an acute fibrinous pneumonia. Extension to the pleural surface (pleuritis) and inflammation of the pericardium (pericarditis) are common secondary complications. Although some animals may have clinical signs of pneumonia, or septicemia, others die acutely without any clinical signs (Percy and Barthold, 2001). Often, the only signs of pneumonia are anorexia and depression. This disease is caused by *P. multocida*. Typically, it occurs in young rabbits 4–6 weeks of age and may follow an outbreak of snuffles. Mortality rates may reach 40% of affected rabbits. The gross lesions are typical of acute or peracute fibrinous pneumonia with anteroventral consolidation of the lungs, pleuritis, and pericarditis. Other findings include lung congestion, petechial and ecchymotic hemorrhages, and peritonitis. Microscopically, the bronchioles and alveoli are filled with heterophils, fibrin, and necrotic debris (Dahlgren, 1992).

Even though it is possible to obtain rabbits that are *Pasteurella* free, pasteurellosis continues to be a significant bacterial disease in rabbits. Infections by *P. multocida* can be manifested in several forms such as snuffles, enzootic pneumonia, otitis media, genital infections (metritis or pyometra and orchitis), abscesses, conjunctivitis, and septicemia. Many rabbits carry this organism without any clinical signs of infection. *P. multocida* is easily transmitted from rabbit to rabbit and can spread from an infected dam to her offspring by the respiratory route shortly after birth. Venereal transmission can occur when genital infections are present. Because it is difficult to recognize infections in carrier animals, an asymptomatic infected rabbit may be introduced with subsequent spread throughout an established colony. These asymptomatic rabbits usually carry the organisms in the nasal cavity. It is believed that stress such as experimental procedures can compromise the rabbit's ability to resist these bacteria allowing them to multiply rapidly and spread to other sites as well as other animals. The development of the specific clinical disease may depend upon the route of infection or extent of dissemination. Other bacterial agents that may cause respiratory disease in rabbits include *S. aureus* and *P. aeruginosa* infections. *Bordetella bronchiseptica* is considered to be a part of the normal bacterial flora in rabbits and in most situations is nonpathogenic. A coronavirus has been identified that causes pleural effusions in rabbits. Clinical signs are fever, anorexia, weight loss, hind limb weakness, and difficult breathing (Richardson, 2000). Gross findings are pulmonary edema and pleural effusions. Microscopic changes in fatal infections were characterized by lymphoid depletion of the spleen follicles, focal degenerative changes in the thymus and lymph nodes, proliferative changes in glomerular tufts, and uveitis. Animals that survive the acute disease have myocardial degeneration and necrosis, focal hepatic necrosis, and proliferative changes in the spleen, lymph nodes, interstitial pulmonary tissue, and glomeruli (DiGiacomo and Mare, 1994).

Metastatic uterine adenocarcinomas are the most frequent neoplastic lesions in the lungs. Clinical signs of affected rabbits include weight loss, dyspnea, and lethargy (Richardson, 2000). Primary neoplasms in the lung are very rare (Weisbroth, 1994).

Endocrine System

Pituitary Gland

The pituitary often contains multiple endothelium-lined spaces at the junction between the pars distalis and intermedia. True cystic spaces or hyperplasia are not common in this species. An eosinophil adenoma has been reported in the anterior pituitary (Weisbroth, 1994).

Adrenal Gland

The adrenal glands are paired organs located anterior and medial to the kidneys. They appear flattened against the dorsal body wall. The left adrenal is adjacent to the origin of the superior mesenteric artery, and the right adrenal lies adjacent to the postcava. Adrenal glands have the usual cortex areas with a distinct medulla seen in other species. The cortex often contains vacuolated cytoplasmic areas in cells that compromise the zona fascicularis. Accessory cortical nodules are found beneath the cortical capsule or embedded within the adipose tissue adjacent to the capsule. Greene (1965) reported that localized areas of adrenal cortical hyperplasia resembling adenomas occurred in his rabbits associated with the coming of spring. These hyperplastic lesions disappeared as the seasons changed. The adrenal medulla may contain areas of hyperplasia involving small groups of cells. While these foci may suggest preneoplasia, there is no reported evidence that pheochromocytomas develop from these hyperplastic foci. Two adrenal cortical carcinomas have been reported in rabbits (Weisbroth, 1994).

Thyroid and Parathyroid Glands

The bilobed thyroid gland is located in the usual lateral aspect of the trachea, distal to the cricoid cartilage. Microscopically, the parathyroid is often embedded with the body of the thyroid. The thyroid contains follicles of various sizes that contain colloid. Typical C cells are difficult to identify by standard light microscopy. Thyroids of adult animals often contain lymphoid aggregates within the interstitial areas. Ectopic thymic tissue can be identified within the capsule of the thyroid. The parathyroid presents no unusual changes with advancing age.

Urinary System

Kidney

The overall kidney structure is similar to that of the rat. It is bean shaped. On longitudinal section, a single papilla has extensive evaginations of the pelvic fornices. The number of glomeruli increases after birth. About 60 ectopic glomeruli are present in each kidney of adult rabbits. Aging glomerular changes begin to be observed at 1 year of age. These changes consist of mesangial proliferation with a multifocal distribution. Progression to a more diffuse distribution and extension into the capillary loops is the end stage of this mesangial proliferation. The capillary wall appears to thicken with age, but the exact nature of this thickening has not been well documented. Numerous experimental studies exploring the histological and ultrastructural changes in immune complex glomerulonephritis have shown a deposition of immune complexes at varying locations along the capillary wall. It is not known if such immune complex disease(s) occur in the rabbit in a natural setting. It is not unusual to find obsolete glomeruli or glomeruli that contain peripheral capillary loops that are collapsed or adherent to each other. There are no other changes and clinical chemistry and urinalysis values do not suggest renal disease. Perhaps ultrastructural changes occur, but such reports have not been forthcoming. Amyloid and hyaline material containing lipid has been found within capillary configurations. These deposits have been found in animals fed high-lipid diets and used in cholesterol-related studies.

Observations of evidence of kidney disease are fairly common during necropsies of old rabbits. Although renal failure is rare, a history of polydipsia, polyuria, or urine scald in a depressed, thin aged rabbit should suggest advanced renal disease. Kidney damage can be caused by infections by *E. cuniculi*, *S. aureus*, and *P. multocida*.

Tubular and interstitial changes consist of the deposition of pigments or lipids and lymphoid and macrophage infiltration. Lipid droplets are often found in the cytoplasm of the convoluted tubular epithelium in animals that have been fasted prior to sacrifice. Other pigments have been found in the convoluted and/or collecting tubules and usually represent products associated with red cell destruction or hepatic failure. Focal or multifocal areas of interstitial mononuclear cell infiltration are common findings. Usually, there is no evidence of tubular damage or evidence of specific infectious agents. However, leptospirosis has been suggested as a possible etiological agent, but there has been no documented case in the recent English literature. The cause of these mononuclear infiltrates remains undetermined.

Embryonal nephromas are relatively common spontaneous neoplasms in rabbits (Weisbroth, 1994). Experimentally, nephroblastomas can be produced in large numbers by exposure to N-ethylnitrosourea (Haenichen and Stavrou, 1979).

Urinary Bladder

Urine is normally cloudy due to the large amounts of crystals. The normal color may range from yellow to red or red brown. The pH is typically alkaline. The urinary bladder in mature rabbits

contains alkaline urine having abundant quantities of dull yellow to brown calcium carbonate and triple phosphate crystals (ammonium magnesium and calcium carbonate monohydrate). In rabbits, the calcium is absorbed in proportion to the amount in the diet. Excess calcium and magnesium are excreted primarily in the urine. Occasionally, the urine of normal rabbits has a dark red to orange color. This color is thought to be due to dietary porphyrins. Pigmented urine has also been associated with elevated levels of urobilin.

Adult pregnant females may develop urinary retention due to the position of the uterus. Occasionally, microscopic examination of the urinary bladder will reveal acute suppurative inflammation of the mucosa (cystitis). Frequently, this cystitis will extend into the ureter (ureteritis) and renal pelvis (pyelitis). The pyelitis can lead to pyelonephritis and pyonephritis.

The blood in the urine, hematuria, is observed in animals with uterine adenocarcinomas, uterine polyps, episodic bleeding from endometrial venous aneurysms, cystitis, urinary bladder polyps, pyelonephritis, and renal pelvic hemorrhage (Percy and Barthold, 2001).

Genital System

Inguinal canals that connect the abdominal cavity to the inguinal pouches do not close in the rabbit. The inguinal pouches are blind structures located lateral to the genitalia in both sexes. These pouches often accumulate white to brown secretions produced by scent glands in their walls. The male secondary sex glands, i.e., seminal vesicles, vesicular glands, prostate, and bulbourethral glands, are not separable except in general terms.

Does reach puberty at 4–9 months of age and their breeding life span is 3–4 years. The placenta is hemochorial, in which maternal blood flows into sinus-like spaces where the transfer of nutrients to fetal circulation occurs. Passive immunity is transferred to the young via the yolk sac prior to birth rather than by colostrum or the placenta.

As noted earlier, rabbits are very sensitive to heat. Does near kindling and young rabbits in nesting boxes are particularly prone to temperature extremes. Hyperthermia can also cause abortion and a reduction of male fertility.

The rabbit is also the nonrodent species most frequently used to evaluate developmental toxicity. In the 1960s, the drug thalidomide was tested and shown to be safe in rats, but caused severe birth defects in humans. When given to pregnant rabbits, this drug caused fetal malformations, and now many governmental agencies require that chemicals must be tested for developmental toxicity in both rodent and nonrodent species (Anderson and Henck, 1994). Vitamin A imbalances can result in hydrocephalus, infertility, abortions, resorption, and increased neonatal mortalities (Richardson, 2000). Vitamin E deficiencies can cause infertility, muscular dystrophy, prenatal mortalities, and seminiferous tubule degeneration (Cheeke, 1994). Vitamin A deficiencies are a cause of hydrocephalus in rabbit (Newberne, 1973). Large doses of vitamin A given to pregnant rabbits can produce a high incidence of gross congenital malformations (Shenefelt, 1972).

Skin lesions of rabbit syphilis, treponematosis, primarily affect the external genitalia and perineal region but also are found on the chin, nose, lips or mouth, eyelids, and regional lymph nodes. These lesions may be raised, crusted, or ulcerated. They result from bacterial infections caused by *T. cuniculi*. This is a rabbit venereal disease that spreads among adult rabbits during breeding and is transmitted to young rabbits at the time of parturition or lactation. Clinical disease is not common. Transient infertility may occur in both sexes. Infertility has been attributed to metritis or retained placentas in females and to preputial inflammation in males. More often, infected animals are detected by serological testing. Treponematosis has been confused with hutch burn. Also, the skin lesions can resemble those of ear mites or sarcoptic mange.

Genital infections are commonly associated with pasteurellosis and salmonellosis causing metritis and pyometra in females and orchitis and epididymitis in males. These infections occur

more frequently in does, but they also are observed in young rabbits and adult bucks. They often result in a reduction in fertility (Dahlgren, 1992).

Pregnancy toxemia is a problem in obese rabbits and can occur in males and nonpregnant does.

Ovary and Oviducts

The ovaries are slightly elongated, small structures and are frequently obscured by a mass of fat that should be carefully removed for examination. Microscopically, the ovaries are similar in appearance to other species. An enlarged portion of the oviduct forms a funnel-shaped structure, the ostium tubae that partially surrounds the ovary. The other end of the oviduct opens into a horn of the uterus.

Ovarian abscesses commonly caused by *P. multocida* are often found during gross and microscopic examinations at the end of reproductive or teratology studies. These abscesses usually contain a thick, tan or white creamy exudate and may be surrounded by a fibrous capsule.

It is not unusual to find oviductal "cysts" on one or both oviducts. Microscopic examinations of these cysts suggest ductal origin. According to Arey (1965), the cranial portion of the mesonephric duct persists as the duct of the epoophoron and its tip becomes the cystic vesicular appendage. These cysts could be termed mesonephric or Wolffian duct cysts.

Ovarian tumors are rarely reported in older female rabbits (Weisbroth, 1994). Affected rabbits may have a history of decreased reproduction rate or failure to conceive.

Uterus and Vagina

The doe has two separate uterine horns with two separate cervices that unite to form the vagina. The uterine horn has a characteristic thick muscular wall. The thickened muscular tissue between the uterus and the vagina is termed the cervix. The urethra lies along the ventral surface of the vagina and joins with the vagina to form a common tube, the urogenital sinus or vaginal vestibule. The external margins of the urogenital sinus form the vulva.

The clitoris is the female homolog of the male penis and lies along the ventral surface of the urogenital sinus. The glans clitoris projects into the urogenital aperture.

Uterine infections, metritis and pyometra, are commonly caused by *P. multocida*. A vaginal discharge is usually the only clinical sign of acute or subacute metritis and pyometra in the doe. Reduced fertility as a result of does that fail to conceive is often observed. The lesions are usually found during gross and microscopic examinations at the end of reproductive or teratology studies.

Endometrial venous aneurysms can result in persistent vulvar or urogenital bleeding. At necropsy, the endometrium has multiple blood-filled, thin-walled dilated veins and blood clots in the lumen of the uterus. These veins apparently rupture and bleed periodically into the uterus lumen resulting in a bloody discharge or hematuria. They appear to be congenital defects seen in multiparous does (Percy and Barthold, 2001).

Bloody vulvar discharge with anorexia, weight loss, and reproductive failure have been observed in animals with uterine hyperplasia and adenocarcinomas. Labored breathing can be a sign of metastasis to the lungs by adenocarcinomas. Cystic mammary glands frequently accompany these uterine changes. Uterine adenocarcinomas are common spontaneous, highly invasive neoplasms that occur in does 2 years of age or older (Baba and von Haam, 1972). The incidence rate of uterine hyperplasia and adenocarcinomas in female rabbits has been reported as high as 80% in does over 5 years of age. The incidence appears to be influenced by breed (Greene, 1965). The highest rate is observed in Dutch rabbits with moderately high incidences in the New Zealand White and Californian rabbits (Stein and Walshaw, 1996).

Leiomyomas and leiomyosarcomas have been reported sporadically in female rabbits (Weisbroth, 1994).

Scrotum

The scrotum of the rabbit is hidden beneath the fur cranial to the penis and ventral to the pelvis. This location is unique for placental mammals. The scrotum consists of a sac of skin, connective tissue, and muscle that encloses the testes.

Testes

The testes are paired, elongated, whitish organs with a marbled appearance that are enclosed within a thick sheath, the tunica albuginea. They descend at 12–14 weeks (Richardson, 2000). Spontaneous testicular findings in adult rabbits include perivascular mononuclear cell infiltrations. In some rabbits, there is evidence of vascular damage and inflammation (periarteritis). It is not uncommon for a few peripheral seminiferous tubules in a section of the testes to appear to be aspermatogenic or necrotic. This is most likely a fixation and handling artifact (Dahlgren, 1992).

The most frequent testicular neoplasms are interstitial cell tumors. They occur predominantly in 5–7-year-old bucks used for breeding. These tumors cause enlargement of the testes with areas of necrosis. Seminomas have been rarely reported (Weisbroth, 1994).

Epididymis

The epididymis is a highly vascularized and highly convoluted tubule that is a continuation of the seminiferous tubules from the testes. Interstitial perivascular mononuclear cell infiltrations are common observations in the epididymis, as well as the seminal vesicle, vesicular gland, and prostate.

Inflammation of the testes, orchitis, and the epididymis, that is, epididymitis, in the male can be caused by *P. multocida*. The lesions are usually found during gross and microscopic examinations at the end of reproductive or teratology studies. These infections can cause a reduction in fertility by preventing conception of the does (Dahlgren, 1992). *P. multocida* can be transferred from rabbit to rabbit by males with genital infections. Clinical signs of orchitis and epididymitis in the buck may include enlarged testes or development of abscesses with purulent drainage. These abscesses usually contain a thick, tan, or white creamy exudate and may be surrounded by a fibrous capsule.

Seminal Vesicle

The seminal vesicle is an elongated sac originating from the base of the bladder and lying along its dorsal side. Spontaneous vesicular epithelial squamous metaplasia, hyperplasia, and keratinized nodules were described by Zwicker et al. (1985).

Vesicular Gland

The vesicular gland, also termed the coagulating gland, is a weakly bilobed tubuloalveolar gland adjacent to the prostate and separated by a thin connective tissue septum with smooth muscles.

Prostate Gland

The prostate gland is a glandular thickening of the dorsal wall of the basal portion of the seminal vesicle. Spontaneous prostate epithelial squamous metaplasia, hyperplasia, and keratinized nodules were described by Zwicker et al. (1985).

Bulbourethral Gland

The bulbourethral gland is a small structure lying on the dorsal surface of the urethra behind the prostate gland.

Penis

The penis consists of a pair of heavy muscles, the crura, a body formed by the corpora cavernosa, and the end, the pars libera. The pars libera is protected by a fold of skin, the prepuce.

Cardiovascular System

The heart is similar to other species except that it is relatively small and the right atrioventricular valve is bicuspid rather than tricuspid. The right chambers of the heart are relatively thin walled, and a frequent postmortem finding is a quantity of clotted blood in the right ventricle, with no evidence of postmortem contraction (Percy and Barthold, 2001). Veins of rabbits are delicate and thin walled and tear easily (Harkness et al., 2010).

Pale streaks running parallel to the long axis of the ventricle are frequent gross findings in obese females and in females late in gestation (26–30 days). These streaks are on the epicardial surface and can be confused with pericardial fat unless the pericardial sac is removed. Microscopically, the streaks have areas of mineralized fibers or a loss of fiber staining but no evidence of inflammation. The etiology is unknown.

Spontaneous interstitial myocarditis consisting of irregularly distributed foci of mononuclear inflammatory cells has been frequently reported in apparently healthy adult rabbits. In other reports, small lymphocytic infiltrates with no evidence of myocardial damage or other inflammatory reactions were observed in a very large percentage of clinically healthy and grossly normal rabbits (Lehr, 1965).

Cardiomyopathy has been observed in rabbits. A possible cause is the rabbit coronavirus. This cardiomyopathy is associated with pleural effusions. Clinical signs are those of congestive heart failure including increased respiratory rate and effort with generalized muscle weakness. Gross findings are pleural effusion, a dilated right ventricle, and pulmonary edema.

A systemic viral disease has been reported in Europe and China. The cause is considered to be a calicivirus that causes a hemorrhagic disease. The course of the disease is acute with high morbidity and mortality. When observed, clinical signs are depression, anorexia, fever, and incoordination that can progress quickly to death. Pathologic findings are hepatic necrosis and hemorrhages in the lungs, kidneys, and other tissues.

Both arteriosclerosis and atherosclerosis occur in rabbits. Arteriosclerosis is common in several breeds. Spontaneous arteriosclerosis occurs in the aorta of rabbits, especially in the arch (Lehr, 1965). Vitamin D, calcium, and phosphorus imbalances, especially hypervitaminosis D, have been associated with arteriosclerosis in rabbits. Clinical signs have included anorexia, dehydration, and weight loss. One affected rabbit had seizures. Gross and microscopic examinations reveal mineralization at the aortic arch and in the thoracic aorta. Atherosclerosis occurs as a result of hereditary hypercholesterolemia in the Watanabe rabbit. Rabbits of this breed develop severe atherosclerosis and are used as a research model for studying this disease (Kraus et al., 1984; Jayo et al., 1994).

Also, rabbits provide a useful model for the study of Purkinje fibers in the heart (Brewer and Cruise, 1994).

When a ketamine/xylazine combination or the alpha agonist, detomidine, was administered to rabbits, multifocal myocardial degeneration with interstitial fibrosis was observed. The cause is attributed to ischemia secondary to vasoconstriction and a reduction in coronary blood flow.

The myocardium of rabbits has limited collateral circulation. Early lesions have degeneration of myofibers with mononuclear and polymorphonuclear cell infiltrations. Late lesions have loss of myofibers and marked interstitial fibrosis (Percy and Barthold, 2001).

Hematopoietic and Lymphatic System

The rabbit has been an important experimental animal in the study of immunology and infectious agents. They were very valuable in the development of a cure for diphtheria (Arrington, 1978). Rabbits are an important source for the production of polyclonal antibodies (Stills, 1994). A strain of rabbits deficient in the sixth component of complement inherit this defect as an autosomal recessive trait (Kraus et al., 1984).

When transported from the breeding rabbitry to a testing or research facility, young rabbits experience extensive stress during shipment and upon arrival including variations in temperature, unusual noise, and unfamiliar handling. Toth and January (1990) have shown the effect of shipment on various hematological and physiological parameters affecting adult male New Zealand White rabbits.

Systemic disease involving the blood and multiple organs, especially those of the lymphoid tissues, includes *P. multocida*, *S. aureus*, *S. typhimurium*, *S. enteritidis*, and *Toxoplasma gondii*. Septicemia has been often associated with snuffles and pneumonia caused by *P. multocida*. Affected animals die peracutely with no evidence of clinical disease and few, if any, gross or microscopic changes of the infection. The diagnosis is determined by isolation of the organism (Richardson, 2000). Other organisms that may result in septicemia are *S. aureus* and *B. bronchiseptica*.

Hypercalcemia is a frequent complication of neoplastic disease. The transplantable VX-2 carcinoma of rabbits provides a model for studying mechanisms of osseous-mediated hypercalcemia in the absence of skeletal metastases (Young et al., 1978).

Blood and Bone Marrow

Neutrophils and lymphocytes are present in blood smears in approximately equal numbers (30%–70%). Basophils occur more commonly than in most mammals (2%–7%) (Harkness et al., 2010).

The rabbit neutrophil contains eosinophilic granules and is commonly referred to as the heterophil or pseudoeosinophil. In suppurative lesions, it is often mistaken for the similarly appearing eosinophil, although the heterophil is distinguished by smaller, lighter stained granules. Hyposegmented neutrophils can occasionally be observed in blood smears. This is known as the Pelger–Huet anomaly. This anomaly is inherited as a partial dominant trait in rabbits.

Clinical biochemistry and hematology values are presented by several sources with emphasis on normal or untreated rabbits. Detailed information is available in McLaughlin and Fish (1994). Other sources are Fox and Laird (1999) and Loeb and Quimby (1999).

Spleen

The spleen is a flat, elongated, dark red structure on the left dorsolateral surface of the greater curvature of the stomach. The microscopic anatomy is similar to that of mice and rats.

Thymus

The thymus persists into adult life, so it is usually large in adult rabbits. It lies somewhat ventral to the heart and extends forward into the thoracic inlet. The microscopic anatomy is similar to that of mice and rats. Thymomas occur infrequently in mature rabbits.

Lymph Nodes

Lymph nodes are bean-shaped structures spread throughout the body and connected to a system of the lymph vessels. Nodes beneath the skin and between muscles are bilateral. These include the cervical, axillary, brachial, inguinal, and sciatic nodes. Most visceral lymph nodes are not bilateral but a few associated with paired organs. These include the mediastinal, pancreatic, renal, mesenteric, and lumbar. The microscopic anatomy is similar to that of mice and rats. The lymph nodes have a fibrous outer capsule that extends trabeculae into the node and subdivides it. A subcapsular sinus communicates with the lymphatic system. Blood vessels and nerves enter and efferent lymph vessels and veins drain from the hilus. The node consists of a peripheral cortex, paracortex, and medulla. The cortex consists largely of lymphoid follicles including germinal centers. The paracortex is lymphoid tissue between the follicles of the outer cortex and the medulla. The medulla consists of sinuses and cords of lymphoid cells.

Lymphosarcoma is the most common tumor of juvenile and young adult rabbits. There may be a genetic predisposition in this species. Clinical signs include lethargy, anorexia, weight loss, and pale mucous membranes. Most affected rabbits have the visceral form involving the liver, spleen, kidney, mesenteric lymph nodes, stomach, adrenal glands, lungs, and bone marrow.

Nervous System

Torticollis that is twisting of the neck is occasionally seen in rabbits. Affected rabbits may also have inappetence and ataxia. The usual cause is infections of the middle or inner ears by *P. multocida*. Other causes are encephalitozoonosis, encephalitis, or mechanical injuries of nervous tissues by migration of aberrant parasites, such as *Baylisascaris procyonis*.

B. procyonis is a common parasite of the raccoon. When rabbits consume hay or bedding contaminated with raccoon feces containing infective *B. procyonis* eggs, larvae are released in the intestine. Because these larvae are in an unnatural host, they aggressively migrate through the lungs and various other organs, especially the central nervous system (CNS). They cause devastating cerebrospinal damage. Clinical signs of this damage include torticollis, ataxia, circling, opisthotonus, and recumbency with fatal results. Gross findings often include multiple, small circumscribed, and raised white nodules in the subepicardial and subendocardial surfaces of the heart and the serosa of the liver. Microscopic examinations of these nodules reveal focal granulomatous inflammation with infiltrations of mononuclear cells and heterophils. Parasite remnants are usually present. Lesions in the CNS consist of malacia and astrogliosis where the larvae have migrated. These areas have gitter cells and a variety of inflammatory cells including lymphocytes, macrophages, eosinophils, and heterophils. Frequently, nematode larvae can be identified in the adjacent tissues (Percy and Barthold, 2001). Visceral larval migrans can also result from infections by *B. columnaris*, a skunk ascarid, and *Toxocara canis*, a dog ascarid, if rabbits are exposed to contaminated feed and bedding.

Encephalitozoonosis is an infection of rabbits caused by the protozoan *E. cuniculi*. This protozoan usually causes latent infections with no clinical signs of a CNS disease. However, tremors, ataxia, paresis, head tilt, and convulsions have been reported in affected animals. Usually, the disease is discovered, when granulomas are observed in brain sections, most often the cerebrum, from animals that displayed no clinical signs. This protozoan also infects renal tubular epithelial cells (Flatt and Jackson, 1970; Shaddock and Pakes, 1971). Vertical transmission through infective urine is strongly suspected.

Focal meningeal aggregates of lymphocytes are often found in routine sections of the brain and the spinal cord of clinically healthy rabbits. Similar aggregates have been observed in the optic nerve as it leaves the globe.

Otitis media infections occasionally can extend from the middle ear to the ventral surface of the brain, especially to the area of the cerebellum.

Epilepsy has been reported in certain breeds of rabbits with white fur and blue eyes (Okerman, 1994). Vitamin A deficiency is uncommon but can result in hydrocephalus. Vitamin A deficiency in the rabbit provides an animal model for the production of hydrocephalus (Newberne, 1973). Hypervitaminosis A can result in numerous malformations including anencephaly and spina bifida (Shenefelt, 1972). Methylmercury poisoning in the rabbit provides an animal model of this disease that causes lesions of the CNS, especially in the cerebellum, similar to those in man (Koller, 1978).

Special Senses System

Ear

Ears of rabbits are large, highly vascularized organs that serve for heat regulation as well as in sound gathering. They are fragile and sensitive to trauma (Harkness et al., 2010).

Otitis externa is a manifestation of the ear mite, *Psoroptes cuniculi*. (See the section "Skin" for more information.) These mites can produce marked inflammation. Affected rabbits are often observed shaking their heads or scratching their ears. The inner surface of the external ear may be filled by foul-smelling bran-like tan or brown crusty exudates. The ear is often thickened and edematous.

Otitis media is inflammation of the inner ear that is clinically characterized by ataxia, head tilt, wry neck, or torticollis. Usually, it is the result of an infection caused by *P. multocida* that has spread from the nasal cavities. Other bacterial organisms that have been implicated are *S. aureus* and *B. bronchiseptica*. Many rabbits with otitis media are asymptomatic. Lesions consist of inflammatory exudate in the tympanic cavities. Occasionally, the infection can extend to the ventral surface of the brain.

Eye

A very wide field of vision reaches 190° for each eyeball. Wide papillary dilation results a light sensitivity approximately eight times greater than humans (Harkness et al., 2010).

Ocular irritation studies are routinely performed using rabbits (Kraus et al., 1984; Anderson and Henck, 1994). Although the eye is used in many toxicological studies, only gross or clinical observations are frequently recorded (Dahlgren, 1992). Microscopic changes often involve the conjunctiva and cornea and consist of multifocal areas of cellular infiltrates and necrosis of the superficial and deep corneal epithelium. Lymphoid infiltration is more common in the conjunctiva. The cellular infiltrates are an admixture of heterophils and macrophages. Corneal erosions may progress to ulceration, the final stage of many eye lesions. Sections of the globe may reveal anterior and/or posterior synechiae. The filtration angle is often difficult to visualize with H&E-stained section; however, the presence of inflammatory cells in the ciliary body or the base of the iris may contribute to the misconception of increased ocular tension. While the choroid lacks pigment, it is not unusual to find foci of melanin-like pigment that appears to extend into the layer of rods and cones of the retina. This pigment does not appear to be the result of inflammation.

Toxic effects of 6-aminonicotinamide were observed in eyes of rabbits given intraperitoneally cumulative doses from 13 to 49 mg/rabbit. The principal lesions were diffuse, bilateral cytoplasmic vacuolation of the iridial and ciliary epithelium in male and female albino and pigmented rabbits (Render and Carlton, 1991).

Conjunctivitis, possibly caused by the bacteria entering the conjunctival sac via the nasolacrimal duct, frequently occurs in both young and mature rabbits. Clinical signs of conjunctivitis are reddened conjunctivae, moderately swollen or closed eyelids, and a serous, mucus, or purulent

discharge (Flatt, 1974). If chronic, the discharge can cause a loss of hair on the face below the medial canthus. The inflammation may be primary or secondary as part of acute or chronic respiratory disease. The organism most frequently isolated is *P. multocida*. This organism can be present in grossly normal eyes. Subsequent ocular irritation studies may be compromised by the development of the inflammatory response to this organism.

Entropion, either from congenital defects or from inflammation, can damage the conjunctiva and the cornea. Affected rabbits have blepharospasm, conjunctivitis, epiphora, and corneal ulceration depending upon the severity of the entropion.

Buphthalmia is a condition in which the globe of the eye is enlarged and distended. An autosomal recessive inherited defect impairs the drainage of aqueous humor from the anterior orbital chamber, primarily in New Zealand White rabbits. The disease may be bilateral or unilateral with various times of onset. The increased intraocular pressure causes changes in the shape and size of the eye, resulting in clinical enlargement and corneal opacity. The cornea has a bluish or cloudy appearance. Conjunctivitis often results from trauma and drying of these large eyes (Suckow and Douglas, 1997).

Rabbits serve as useful models for ophthalmology and vision research (Peiffer et al., 1994).

Corneal dystrophy, cataracts, and intraocular tumors are reported infrequently in the rabbit.

A malignant melanoma of the eye that infiltrated the choroids, iris, and ciliary body has been described (Weisbroth, 1994).

The rabbit has a well-developed nictitating membrane, the third eyelid, a semilunar fold at the anterior angle of the eye. During sleep, the third eyelid moves across the cornea.

Harderian Gland

The Harderian gland is a large bilobed structure located behind the third eyelid. Swelling and protrusion of the third eyelid are associated with the Harderian gland. Affected animals have unilateral or bilateral large red masses protruding from the third eyelid at the medial canthus of the eye. Possible causes of this protrusion are inflammation of the Harderian gland (Richardson, 2000) or an abnormal laxity of the connective tissue attaching the Harderian gland to the bony orbit (Percy and Barthold, 2001).

Nutrition and Metabolic Diseases

Malnutrition is uncommon in rabbits. Rabbits, especially adults, are very sensitive to vitamin D toxicity. This toxicity results in calcification of many organs and tissues. Relatively uncommon nutritional problems include vitamin D, calcium, and phosphorus imbalances (atherosclerosis), vitamin A (hydrocephalus, prenatal death) and vitamin E (muscular dystrophy, prenatal mortality, seminiferous tubule degeneration) deficiencies, and some specific mineral or amino acid deficiencies (Cheeke, 1994). Vitamin A deficiencies are a cause of hydrocephalus in rabbit (Newberne, 1973). Large doses of vitamin A given to pregnant rabbits can produce a high incidence of gross congenital malformations (Shenefelt, 1972).

Bacterial, Mycotic, and Viral Diseases

For detailed descriptions of bacterial diseases and the causative microorganisms, see Flatt (1974), Gleiser (1974), and DeLong and Manning (1994). Mycotic infections occur but are less frequent in rabbits; see Bergdall and Dysko (1994). Viral diseases and the causative agents are described in detail by DiGiacomo and Mare (1994). Use of rabbits as models in infectious disease research is presented by Fox et al. (1994). The impact of bacterial, mycotic, and viral infections on research studies is discussed by Baker (1998) and Baker (2003).

Parasitic (Protozoan and Metazoan) Diseases

For detailed descriptions of protozoal diseases and the causative organisms, see Pakes and Gerrity (1994). Arthropod and helminth parasites are described by Hofing and Kraus (1994). The impact of parasites on research studies is discussed by Baker (2003).

Neoplastic Diseases

Weisbroth (1994) reviewed the literature very thoroughly for neoplasms reported in rabbits, with emphasis on naturally occurring or spontaneous neoplasms, unrelated to treatment. Although the overall incidence of neoplasia is reported to be 2%–3%, this incidence is biased by the high percentage of rabbits at necropsy at ages too young to be in cancer-prone age groups. A normal healthy rabbit has a life span of approximately 6–8 years; however, a few can live up to 15 years (Harkness et al., 2010). Rabbits, as with other species, demonstrate that age is a predominant factor in determining neoplastic incidence. In a report of 849 female rabbits dying of various causes from a colony where animals were permitted to die of natural causes, 16.7% had uterine adenocarcinomas (Greene, 1965). The average age at death was greater than 4 years. In the affected rabbits, uterine adenocarcinomas had a 4.2% incidence rate in females 2–3 years of age and 79.1% in females 5–6 years of age. In a smaller study, the incidence of neoplastic disease was seven times higher after the second year of life. An incidence of approximately 8% was the same for males and females over 2 years of age. Uterine adenocarcinomas in female rabbits between 1 and 2 years of age resulted in an overall tumor incidence that was six times higher in females than males (Weisbroth, 1994). If the life span of the rabbit is considered to be 7 years, the ratio of the rabbit to the human life span would be approximately 1:12. A rabbit of 6 years would be roughly equivalent to 75 years in man, over 90% of the human life span (Peckham, 1980). Some tumors in rabbits have shown a clear genetic predisposition. Other tumors are associated with oncogenic viruses. A few transplantable rabbit tumors are available for research studies. In addition to uterine adenocarcinomas, a wide variety of other neoplasms have been reported. These tumors affect most organ systems, including the uterus, vagina, mammary glands, kidney, urinary bladder, liver, skin, bone, mesenchymal tissues, lymphoid tissues, testes, stomach, intestines, lung, nervous system, and endocrine glands (Weisbroth, 1994). The most commonly occurring spontaneous tumors, in order of frequency, are uterine adenocarcinomas, lymphosarcomas, embryonal nephroma, and bile duct adenoma of the liver. Lymphosarcomas occur most commonly in young rabbits. Rabbits used in "typical" toxicological or teratological studies rarely have spontaneous neoplasms because of the limited duration of these studies.

Conclusions

The rabbit provides useful animal models for toxicology and a broad variety of experimental diseases and research studies in several organ systems. Advantages of the rabbit are that it is hardy, small but large enough to easily observe irritation and evaluate fetuses, relatively clean, inexpensive, and easily housed and handled, requires moderate amounts of test materials, has a short gestation period, and is sensitive to teratogens. Rabbits are good antibody producers and blood is relatively easy to collect. The scientific literature provides ample historical background and reference information for investigators. The rabbit is generally the nonrodent species of choice for studies that evaluate potential adverse effects on reproduction, organ development, and risks for teratologic effects. Other examples of studies routinely using rabbits include ocular, dermal, and mucous membrane irritation, dermal toxicity, toxicokinetic studies, pyrogen testing, intracutaneous implants, neurotoxicity, nephrotoxicity, and immunotoxicity (Anderson and Henck, 1994). Investigators using rabbits must be aware of a few but potential major disadvantages. The microflora of the gastrointestinal tract is

easily disrupted, leading to diarrhea and poor health. The diets fed and management are very impor-
tant. Also, the rabbit is highly susceptible to exposure to certain types of antibiotics. The impact
of bacterial, mycotic, viral, and parasitic agents on research studies is discussed by Baker (1998)
and Baker (2003). For additional information on subjects related to the use of the rabbit in research
studies, the reader is referred to the comprehensive publications edited by Weisbroth et al. (1974),
Hillyer and Quesenberry (1997), and Manning et al. (1994). Some specific subjects are covered in
the following references. Anatomy is described by Cruise and Brewer (1994) and McLaughlin and
Chiasson (1979), and details are illustrated in an atlas by Popesko et al. (1992). Dissection instruc-
tions are given by Wells (1968), Wingerd (1985), and McLaughlin and Chiasson (1979). Necropsy of
the rabbit is described by Bivin (1994) and in greater detail by Feldman and Seely (1988). Physiology
is discussed by Kaplan (1962), Kozma et al. (1974), Kaplan and Timmons (1979), and Brewer and
Cruise (1994). Clinical chemistry and hematology values are presented in detail by McLaughlin
and Fish (1994), Fox and Laird (1999), and Loeb and Quimby (1999). General husbandry and dis-
ease management including clinical descriptions of common and uncommon diseases with possible
treatments are presented by Whitney (1979), Holmes (1984), Adams (1987), Harkness et al. (2010),
Stein and Walshaw (1996), Suckow and Douglas (1997), and Richardson (2000). Pathology find-
ings are presented in Benirschke et al. (1978) and described in detail by Percy and Barthold (2001).
Weisbroth (1994) has completed a comprehensive review of neoplastic findings in the rabbit. Other
references on neoplasia in rabbits include Squire et al. (1978) and Stedham (1976).

METABOLISM

Shayne Cox Gad

Hepatic microsomal cytochrome P-450 (CYP-450) activity in the rabbit has been studied and com-
pared with those in other laboratory animals such as the mouse, rat, hamster, and guinea pig (Davies
et al., 1969; Chhabra et al., 1974; Litterst et al., 1975, 1976), as have the levels and activities of its sub-
strates. The CYP-450 content (1.05–1.09 nmol/mg) and the activity of NADP–cytochrome c reductase
(130–150 nmol/min/mg) in the rabbit were similar to those in the other species examined (Table 6.5).
While CYP-450 reductase activity is much lower in the rabbit (3.0–3.4 nmol/min/mg) compared with
that in the mouse and rat, the full range of CYP-450 isoenzyme activities is present (Table 6.6).

Souhaili-El Amri et al. (1986) conducted a similar study in six laboratory animal species and
humans. Although the values for the CYP-450 concentrations were somewhat lower in this study
compared with those in the previous studies, the value in the rabbit (0.67 nmol/mg) was generally
similar to those in the other animals, which is consistent with earlier findings. The CYP-450 content
in the rabbit was somewhat higher than the value in humans (0.31 ± 0.09 nmol/mg).

Studies of rabbit CYP-450 using SDS-acrylamide gel electrophoresis have demonstrated at least
15 separable forms from liver microsomes other than P-450. The PB-induced form, P-450 LM_2,
and BNF/3-MC-induced form, P-450 LM_4, are the most active, although all of the P-450 isozymes
displayed enzymatic activities. P-450 LM_2 was most effective in the metabolism of benzphetamine,
whereas P-450 LM_4 was specific for acetanilide hydroxylation. However, they displayed comparable
activity toward p-nitrophenetole, p-nitroanisole, and 7-ethoxycoumarin. The isozyme LM_3, which is
similar to CYP-450$_p$ in the rat, was also found in the rabbit treated with macrolide antibiotics such
as erythromycin and triacetyloleandomycin (Bertault-Peres et al., 1987; Fabre et al., 1988). This iso-
zyme specifically mediates the metabolism of cyclosporin. The isozymes LM5 and LM6 exclusively
mediate the metabolism of 2-aminofluorene and 7-ethoxyresorufin, respectively.

The distribution pattern of mixed function oxidases for xenobiotics between smooth and rough
microsomal membranes is known to be highly species dependent. In the rabbit, concentrations of all
components of the mixed function oxidase system are 4–5 times higher in the smooth endoplasmic

Table 6.5   Summary of Hepatic Xenobiotic Drug-Metabolizing Enzymes in the Rabbit

Enzyme	Concentration or Activity
CYP-450 (nmol/mg protein)	1.1,[a] 0.779,[b] 1.05 for M and 1.09 for F,[c] 0.720,[d] 0.67,[c] 0.81[f,g]
Cytochrome b_5 (nmol/mg protein)	0.3,[e] 0.84[b]
NADPH–cytochrome c reductase (nmol/mg protein)	185,[a] 152 g, 130 for M and 150 for F[c]
NADPH–CYP-450 reductase (nmol/mg protein)	3.0 for M and 3.4 for F[c]
Hydroxylase (nmol/min/mg)	
Aniline hydroxylase	0.65,[a] 0.542,[b] 0.6,[g] 0.72[h]
Benzo(a)pyrene hydroxylase	0.061,[d] 0.11[i]
Biphenyl 4-hydroxylase	3.9,[a] 1.7,[g] 1.1,[f] 2.0[i]
Styrene oxide hydroxylase	5.6,[j] 5.8–6.4,[k] 4.2,[l] 2.3,[m] 10.1[n]
O-dealkylase (nmol/min/mg)	
Ethoxycoumarin O-dealkylase	2.3[f]
P-nitroanisole O-dealkylase	5.4[f]
N-demethylase (nmol/min/mg)	
Aminopyrene N-demethylase	15.0,[b] 9.8,[g] 8.0[h]
Ethylmorphine N-demethylase	4.0,[a] 4.0 for M and 4.3 for F[c], 2.0–2.2[o]
N-hydroxylase (nmol/min/mg)	
Dibenzylamine N-hydroxylase	2.9[p]
2-Acetylaminofluorene N-hydroxylase	0.2,[f] 0.1[i]
Glutathione S-transferase (nmol/min/mg)	
1-Chloro-2,4-dinitrobenzene	4091[n] (cytosol), 81[n] (microsomes), 156[q] microsomes
Hexachloro-1,3-butadiene	0.14[n] (microsomes)
Ethacrynic acid	5.81[r]
Styrene oxide	30.5,[j] 21.3–26.9,[k] 36[l]
Protein estimates (mg/g)	
Microsomal	24.1,[g] 13.8–22.8,[k] 18.1[d]
Cytosolic	72.7–82.0,[k] 86.5[d]

[a] Chhabra et al. (1974).
[b] Oppelt et al. (1970).
[c] Davies et al. (1969).
[d] Gregus et al. (1983).
[e] Souhaili-el Amri et al. (1986).
[f] Atlas et al. (1975).
[g] Thorgeirsson et al. (1979).
[h] Kato (1979).
[i] Thorgeirsson (1979).
[j] James et al. (1976).
[k] James et al. (1977).
[l] Pacifici et al. (1981).
[m] Oesch (1973).
[n] Oesch and Wolf (1989).
[o] Nerland and Mannering (1978).
[p] Beckett and Gibson (1975).
[q] Morgenstern et al. (1984).
[r] Gregus et al. (1985).

reticulum than in the rough endoplasmic reticulum of the hepatic microsomal fractions (Gram et al., 1971), whereas some animals (e.g., the rat and mouse) showed fairly even distribution between the smooth and rough membranes.

As reported for other species, the drug-metabolizing enzymes in newborn rabbits are not fully developed. For example, phenobarbital was more toxic to newborn than to adult rabbits and produced longer loss of the righting reflex in newborn rabbits (Weatherall, 1960).

Table 6.6   CYP-450 Isoenzyme Activities in the New Zealand White Rabbit

Activity	CYP-450 Isozyme
7-Ethoxyresorufin O-dealkylation	1A1/2[a]
7-Methoxyresorufin O-dealkylation	1A2[a]
Caffeine 3-demethylation	
Benzphetamine N-demethylation	2B4/5[b,c]
7-Benzoxyresorufin O-dealkylation	2B4/5[b]
7-Pentoxyresorufin O-dealkylation	2B4/5[b]
Coumarin 7-hydroxylation	2A10/11[d,e]
7-Ethoxy-4-trifluoromethylcoumarin demethylation	2B4[f]
Ethoxycoumarin O-dealkylation	2B4/5, 2C3[b,g]
Tolbutamide methyl hydroxylation	3A6[h]
Chlorzoxazone 6-hydroxylation	
4-Nitrophenol hydroxylation	2E1/2[i]
N-Nitrosodimethylamine N-demethylation	2E1/2[i]
Androstenedione 15α-hydroxylation	2B5[b,g]
Androstenedione 16α/β-hydroxylation	2B4/5[b,g]
Dextromethorphan O-demethylation	
Dextromethorphan N-demethylation	
Testosterone → androstenedione	2A10/11[d]
Testosterone 2α-hydroxylation	
Testosterone 2β-hydroxylation	
Testosterone 6β-hydroxylation	2C3, 3A6[a]
Testosterone 7α-hydroxylation	
Testosterone 15α-hydroxylation	
Testosterone 15β-hydroxylation	
Testosterone 16α-hydroxylation	2B4, 2C3[a]
Testosterone 16β-hydroxylation	2B
Lauric acid 11-hydroxylation	4A1, 4B1[j]
Lauric acid 12-hydroxylation	4A1, 4B1[j]

[a] Donato et al. (1999).
[b] Grimm et al. (1994).
[c] Adali et al. (1996).
[d] Ding et al. (1994).
[e] Pearce et al. (1992).
[f] Roberts et al. (1997).
[g] Ryan et al. (1993).
[h] Veronese et al. (1990).
[i] Ding and Coon (1990).
[j] Makowska et al. (1992).

The content of CYP-450 and other electron transport chain components are highest in the rabbit liver among the organs examined, being approximately 2.5–5.0-fold higher than those in the lung and kidney (Uehleke, 1969; Oppelt et al., 1970). However, comparison of monooxygenase activity toward various substrates undergoing the same route of metabolism shows considerable variation in the ratio of lung to liver.

Distribution of monooxygenase activities among the tissues is dependent on the substrates employed. For example, when benzopyrene hydroxylase activity was examined in the small intestinal mucosa preparations of the rabbit, mouse, cat, guinea pig, and rat, the rabbit had medium benzpyrene hydroxylase activity in the small intestine, although the hydroxylase activity in the liver microsomes was least active in the rabbit (Hietanen and Vainio, 1973). The UDP-glucuronyl transferase measured using p-nitrophenol as a substrate was highest in the small intestinal

mucosa of the rabbit, although the transferase activity of the rabbit liver was intermediate and similar to the activity of rat and mouse liver.

The concentrations of CYP-450 and the activities of CYP-450 reductase and NADPH–cytochrome c reductase were similar between the male and the female rabbits (Davies et al., 1969) and in the N-demethylation of ethylmorphine and aminopyrine or hydroxylation of phenobarbital (Davies et al., 1969; Testa and Jenner, 1976). An exception is found in rabbit lung metabolic response to ozone that was found to be more sensitive in females (Delaunois et al., 1999).

Strain differences in drug-metabolizing ability could account for varying biological responses to such compounds as hexobarbital, adrenal cortex hormones, and 2-naphthylamine. In contrast to two- to threefold variation in drug-metabolizing ability seen in the mouse and rat strains, up to 20-fold variations were observed between various rabbit strains. California rabbits, wild cottontail, and jack rabbits exhibited the most striking difference, each being fairly deficient in some enzymes. For example, the in vitro hepatic microsomal metabolism rate of hexobarbital and amphetamine was 19 and 8 nmol/mg protein/hour, respectively, in the cottontail rabbit, whereas the metabolism rate of hexobarbital in the New Zealand rabbit was 254 nmol/mg/ hour, and the metabolism rate of amphetamine in the Dutch rabbit was 154 nmol/mg/hour (Cram et al., 1965).

The capacity for aromatic hydroxylation in the rabbit appears to be medium to high in general as illustrated with aniline, biphenyl, and coumarin. After intravenous administration of aniline, about 50% of the dose was excreted as p-aminophenol (Parke, 1960). While 4-hydroxylation of biphenyl was observed in the rabbit as in humans, 2-hydroxylation was not observed. Biphenyl 2-hydroxylation was limited to a few species such as the mouse and hamster. When the in vitro metabolism of N-benzyl-4-substituted anilines was studied using the rabbit, mouse, hamster, and guinea pig, ring hydroxylation was the major pathway in the rabbit and guinea pig, whereas in the mouse and hamster, N-debenzylation was the major pathway (Gorrod and Gooderham, 1987). The rat used both pathways to an equal extent. Coumarin 7-hydroxylase was present in rabbit hepatic preparations but absent in the rat and mouse preparations (Kulkarni and Hodgson, 1980).

In contrast to the aforementioned examples, the aromatic hydroxylation activity in the rabbit was low with benzpyrene, amphetamine, and ethyl biscournacetate. The hepatic benzpyrene hydroxylase activity of the rabbit was two- to fivefold lower compared with that of the rat and mouse (Hietanen and Vainio, 1973; Gregus et al., 1983) and also somewhat lower than that in humans with a variation of about sixfold (Pelkonen et al., 1975). The range of aryl hydrocarbon hydroxylase activity in humans was approximately 20–320 pmol/g liver/min with no sex difference. In the rabbit, ethyl biscournacetate, an anticoagulant drug, was exclusively deesterified and no aryl hydroxylation was observed, whereas in humans and dogs, aryl hydroxylation of the drug was observed.

In the metabolism of amphetamines, aromatic hydroxylation was minor and deamination was the major pathway in the rabbit (Caldwell, 1976, 1981). Only small amounts were excreted as the unchanged drugs. In humans, deamination and unchanged drug excretion were equally important. In addition, substantial amounts of aromatic hydroxylated metabolites were excreted. N-Dealkylation of amphetamines was a minor pathway in humans as observed in the rabbit (Brodie, 1962). Green et al. (1986) have studied amphetamine metabolism using isolated hepatocyte suspension from the rabbit, rat, dog, squirrel monkey, and human livers. As observed in the in vivo studies, rabbit hepatocytes metabolized amphetamine almost exclusively to the products of the oxidative deamination pathway, whereas rat hepatocytes primarily metabolized by aromatic hydroxylation. Metabolism of the drug by the hepatocytes from three other species (dog, monkey, and human) was mixed, but oxidative deamination was somewhat more active than aromatic hydroxylation.

N-Dealkylating activity in the rabbit was highly variable, ranging from low to high depending on the substrate. For example, N-demethylation of benzphetamine by the rabbit was about twofold higher than that in the rat and mouse, about fourfold higher than that in the dog (Gregus et al., 1983), and more than 10-fold higher than that in humans (Souhaili-El Amri et al., 1986). The maximum velocity (V_{max}) of N-dealkylase activity for aminopyrine in the rabbit was slightly lower compared

with the mouse and male rat (SD strain), similar to that of the monkey and female rat, and about twofold higher than that of humans (Souhaili-El Amri et al., 1986). N-Demethylase activity of ethylmorphine in the rabbit was less than half that of the mouse and male rat, similar to that in the dog and female rat, and about twofold higher than that in humans (Gregus et al., 1983; Souhaili-El Amri et al., 1986). In contrast, N-dealkylation of benzylaniline in the rabbit was minor and ring hydroxylation was the major pathway. Similar results were also observed with tolbutamide, an antidiabetic drug. Tolbutamide is metabolized via methyl hydroxylation and N-dealkylation pathways. In rabbits as well as in humans, monkeys, and rats, the methyl hydroxylation was the major pathway (Thomas and Ikeda, 1966; Tagg et al., 1967; Gee and Green, 1984). In dogs, N-dealkylation was the major pathway (Remmer et al., 1964).

N-Hydroxylase activity is predominately mediated via the polycyclic hydrocarbon-inducible cytochrome PI-450 (Felton et al., 1976) and is of great importance for toxicity evaluation. For example, acetaminophen-induced hepatic injury was found to be related to the rates of N-hydroxylation of the drug by the hepatic microsomes (Davis et al., 1974). In the rabbit, N-hydroxylation activity was medium to high as illustrated with 4-aminobiphenyl and 2-acetylaminofluorene (2-AAF). The major metabolic pathway of 4-aminobiphenyl was N-hydroxylation in the rabbit, mouse, guinea pig, and hamster (McMahon et al., 1980). Interestingly, the N-hydroxylase activity in the rabbit was not induced by methylcholanthrene or Aroclor 1254, a potent cytochrome PI-450 inducer, although it was induced by phenobarbital. N-Hydroxylation of 4-aminophenyl in the rat, mouse, and guinea pig was enhanced more than fivefold by Aroclor 1254.

The N-hydroxylase activity of 2-AAF in rabbit liver microsomes was higher than that in the mouse, rat, and guinea pig (Lotlikar et al., 1967). The N-hydroxy metabolite was practically undetectable in the guinea pig, which explains resistance of the guinea pig to hepatoma induction by 2-AAF. When in vitro metabolism of dibenzylamine was studied using hepatic microsomes of the rabbit, rat, mouse, hamster, guinea pig, chick, and cat, N-oxidase activity in rabbit microsomes was highest (86.9 nmol/mg/30 min) and approximately 5 and 7 times greater than the activity of mouse and rat microsomes (Beckett and Gibson, 1975). In vitro metabolism of N-benzyl-4-substituted anilines was studied using rabbit, rat, mouse, hamster, and guinea pig liver homogenates (Gorrod and Gooderham, 1987). In contrast to the findings with dibenzylamine, the rabbit had the lowest N-oxidase activity, which was followed by the rat, mouse, guinea pig, and hamster in increasing order. In the rabbit, ring hydroxylation was the major pathway.

Hepatic microsomal levels of epoxide hydrolase activity in rabbits have been compared with those of humans and several commonly used animal models, including the mouse, rat, guinea pig, and rhesus monkey (Oesch, 1973, 1980). With benzo(a)pyrene 1, 1-oxide as a substrate, the hydrolase activity in the rabbit was similar to that in the rat but approximately half that in humans and guinea pigs. The monkey had the highest activity and the mouse had the least activity. When the activity of epoxide hydrolase was measured with styrene oxide as a substrate, the enzyme activity in rabbit liver (4.2–10.1 nmol/min/mg) was similar to that in rat liver as observed with benzo(a)pyrene 1, 1-oxide but three- to sixfold lower than that in human liver (Pacifici et al., 1981; Gregus et al., 1983; Oesch and Wolf, 1989). As observed with other enzyme activities, the styrene oxide hydrolase activity in the rabbit kidney and lung (1.5 and 0.4 nmol/min/mg) was lower compared with that in the liver.

The N-acetyltransferase enzyme system is important in understanding the toxicity induced by arylamines. This enzyme system can be viewed as a component of activation pathways with respect to arylamine hepatocarcinogenesis and as a component of detoxification pathways with respect to arylamine bladder carcinogenesis, whereas N-hydroxylating enzyme systems can be viewed as components of activation pathways with respect to both arylamine bladder carcinogenesis and arylacetamide hepatocarcinogenesis. The rabbit is known as the best acetylator of aromatic amines and sulfonamide with low arylacetamide deacetylase activity. For example, consistently high levels of N-acetyltransferase activity were observed in the rabbit toward p-aminobenzoic acid, isoniazid,

sulfamethazine, 2-aminofluorene, and O-naphthylamine, whereas the rat and mouse showed markedly different activities depending on substrate (Gregus et al., 1983). When acetylase activity was studied with sulfanilamide (Williams, 1967) and various mono- and dimethoxy-6-sulfanilamidopyrimidines (Bridges et al., 1969) in various species (such as rabbit, human, monkey, and rat), the major metabolites in the rabbit urine were N^4-acetyl derivatives except in the case of the 2,5-dimethoxy compound, which was excreted largely unchanged. The rat also favored the formation of N^4-acetyl derivatives, but overall excreted more unchanged drug than the rabbit. Comparable results were apparent in humans with a lesser degree of N^4-acetylation compared with the rabbit. Rabbits display a genetic polymorphism with respect to acetylation, as do humans. This leads to speculation that the rabbit may be a predictive animal model for population-based acetylation of aromatic amines (Calabrese, 1988).

Glowinski et al. (1978) compared rates of acetylation in both fast and slow acetylator phenotypes in rabbits and humans for seven compounds, sulfamethazine, p-aminobenzoic acid, and five arylamine carcinogens (α-naphthylamine, 8-naphthylamine, benzidine, 2-aminofluorene, and methylene-bis-2-chloroaniline). In general, the fast acetylator rabbits displayed a 10- to 50-fold greater rate of activity than the fast acetylator humans. For all compounds except p-aminobenzoic acid, the acetylation activity for the fast acetylator rabbits was 90- to 580-fold higher than that of the slow acetylator rabbits, whereas in humans, the activity in fast acetylators was approximately 4- to 13-fold higher. Therefore, for some compounds, the levels of acetylation activity in the slow acetylator rabbits were in the range of the fast or slow acetylator humans. For example, the slow acetylator rabbits were very similar to the slow acetylator humans for 2-aminofluorene (0.013 vs. 0.021 μmol/mg/hour) and benzidine (0.016 vs. 0.019 μmol/mg/hour), but more closely comparable to the fast acetylator human for β-naphthylamine (0.28 vs. 0.23 μmol/mg/hour).

Many studies have been conducted to determine UDP-glucuronyl transferase activity in the rabbit. However, the enzyme activity appears to be highly substrate specific and no general conclusion could be drawn. For example, the transferase activity in the rabbit was high toward 1-naphthol, p-nitrophenol, estrone, morphine, and chloramphenicol when compared to the activity in other species such as the mouse, rat, guinea pig, and cat (Gregus et al., 1983). The transferase activity was medium or low toward phenolphthalein, diethylstilbestrol, testosterone, digitoxigenin, valproic acid, and bilirubin. In the rabbit, the concentration of hepatic UDP-glucuronic acid, which is required in the glucuronidation reaction as a glucuronic acid donor, was about half the values of the rat and guinea pig and similar to that of the dog. Therefore, UDP-glucuronic acid did not appear to be a limiting factor for glucuronidation metabolic pathway.

Emudianughe et al. (1978, 1987) studied glucuronidation and other conjugation reactions of radiolabeled naphthylacetic acids. Following 1-naphthylacetic acid administration, the majority of urinary radioactivity (88%) was accounted for by a glucuronide conjugate, whereas only a small amount (6%) of glycine conjugate was present in the rabbit. However, with 2-naphthylacetic acid, the majority of urinary radioactivity was accounted for by amino acid conjugates (glycine, glutamine, and taurine), whereas the glucuronide conjugate represented about 24% of the urinary radioactivity. This was explained by the steric hindrance of 1-naphthylacetic acid for activation in amino acid conjugation (Caldwell, 1981). The amino acid conjugations require that the carboxylic group of the xenobiotic acid should be readily accessible for activation to the essential acyl CoA intermediate.

Interestingly, solubilized rabbit liver microsomes had relatively high UDP-glucuronyl transferase activity toward estrone and G-estradiol but not testosterone (Falany et al., 1983). Glucuronidation observed at the 3-OH position of β-estradiol is 20-fold greater than at the 17-OH position. In contrast, solubilized liver microsomes from female rats possessed approximately fourfold more activity toward testosterone (17-OH) than estrone (3-OH). Rat liver microsomes formed 2.5-fold more β-estradiol 17-glucuronide than 3-glucuronide. The highly substrate- and species-specific UDP-glucuronyl transferase appears to be due to, at least in part, multiple forms of the enzyme.

Sisenwine et al. (1982) studied in vivo and in vitro stereoselective effects in the glucuronidation of oxazepam in various species. They reported that conjugation of S-(+)-oxazepam was favored in rabbits as well as in humans, dogs, rats, and miniature swine. In rhesus monkeys, conjugation of R-(-) isomer was favored. In contrast to oxazepam, glucuronidation of propranolol was stereoselective for (R)-propranolol in the rabbit and for (S)-propranolol in humans and dogs (Yost et al., 1981; Von Bahr et al., 1982). These workers also found that glucuronic acid conjugation of 4-hydroxy-propranolol, a metabolite of propranolol, was not stereoselective. After administration of racemic 2-arylpropionic acid, the formation of the glucuronide was enantioselective for the S(-) isomer in the rat and mouse but showed no stereoselectivity in the rabbit (Fournel and Caldwell, 1986). These findings as a whole demonstrated complex stereoselectivity of UDP-glucuronyl transferases among different species, and broad generalization could not be made for the suitability of animal models for evaluating stereoselectivity of the enzyme.

Hydrolysis of glucuronide conjugates is carried out by the lysosomal enzyme β-glucuronidase, which is present in most tissues, particularly liver, kidney, spleen, intestinal tract, and endocrine and reproductive organs. The level of β-glucuronidase in multiple tissues has been studied in a variety of animal species as well as in humans. When β-glucuronidase activity was measured with phenol-phthalein glucuronide, the enzyme activity in the rabbit liver (5000 µg/g liver/hour) was about 1.7-fold higher than that of human liver, similar to that of guinea pig and hamster liver, and three- to sixfold lower than that of rat liver (Calabrese, 1988). In contrast to liver enzyme activity, the enzyme activity in the kidney was lower in the rabbit (300 µg/g liver/ hour) than in humans (2000 µg/g liver/hour).

Gastrointestinal tract levels of β-glucuronidase activity may markedly affect the response to some carcinogenic agents. Many carcinogenic agents such as benzo(a)pyrene are conjugated with glucuronic acid and excreted via the bile. Low 6-glucuronidase activity in the small intestine is expected to reduce enterohepatic circulation, thereby reducing the residence time of the carcinogens in the body. Rabbits are estimated to have approximately 120- and 50-fold higher β-glucuronidase activity in the proximal and distal small intestine, respectively, compared with humans (Calabrese, 1988). Mice and rats are estimated to have approximately 60,000- and 15,000-fold, respectively, higher β-glucuronidase activity in the proximal small intestine than humans. Such findings suggest that the rabbit model may offer a closer approximation to the human even though the rabbit far exceeded the human values.

Glutathione transferase activity in rabbit liver was highly variable depending on the substrate. For example, glutathione transferase activity toward 1-chloro-2,4-dinitrobenzene was approximately five- and twofold higher in the rabbit than in the rat and mouse, respectively (Gregus et al., 1983; Igarashi et al., 1986). However, toward 1,2-dichloro-4-nitrobenzene, the enzyme activity in the rabbit was less than one-sixth of the activity in the rat and mouse. Gregus et al. (1985) studied glutathione transferase activities in the 60-day-old rabbit and other commonly used laboratory animals, such as rat, mouse, and guinea pig, toward seven substrates. The rabbit was the least active in the conjugation of six substrates (3,4-dichloronitro-benzene, sulfobromophthalein, p-nitro-benzyl chloride, ethacrynic acid trans-4-phenyl-3-butene-2-one, and 1,2-epoxy-3-(p-nitrophenoxy propane)) out of seven, yet it was the most active in the conjugation of 1-chloro-3,4-dinitrobenzene. The transferase activity toward styrene oxide in rabbit liver (36 nmol/min/mg) was two- and four-fold lower than that in rat and mouse liver but about 1.9 and 1.4 times higher than that in dog and human liver (Pacifici et al., 1981). This high variation of the enzyme activity appears to be in part due to different substrate specificities and subunit composition of the enzymes among the different species (Igarashi et al., 1986). The glutathione transferase activities toward styrene oxide in the rabbit kidney and lung (18.0 and 6.5 nmol/min/mg, respectively) were lower than that in the liver (Pacifici, 1981).

Oesch and Wolf (1989) determined glutathione transferase activity in liver microsomes and cytosol from various species, including rabbits and humans. The glutathione transferase activity toward the microsomal specific compound, hexachloro-1,3-butadiene, in rabbit liver microsomes

(0.14 nmol/min/mg) was about one-tenth of that in human liver. The transferase activity toward the cytosol enzyme-specific compound, 1-chloro-2,4-dinitrobenzene, in rabbit liver cytosol fractions (4091 nmol/min/mg) was more than twofold higher than that in human liver cytosol fractions.

When perinatal development of glutathione S-transferase and epoxide hydrolase was studied in rabbit liver and extrahepatic organs, the activity of glutathione S-transferases toward 1,2-dichloro-4-nitrobenzene and styrene oxide was different in liver, intestine, lung, and kidney (James et al., 1977). In the liver, which has higher activity compared with the other organs, the transferase activity toward both substrates was within the adult range at 70 days after birth. The transferase activity toward styrene oxide in fetal rabbit liver was 1–2 nmol/mg/min. This activity increased sharply and reached about 50% of the adult value between 1 and 6 days after birth. However, the age development of glutathione transferase activities in rabbits was highly substrate specific (Gregus et al. 1985). For example, the glutathione transferase activity in rabbit liver for ethacrynic acid increased only 1.8-fold from the neonatal value during the first 120 days after birth, whereas the enzyme activity toward 1,2-epoxy-3-(p-nitrophenoxy)-propane increased about 18-fold.

Depletion of glutathione in the liver was found to be directly correlated with acetaminophen hepatotoxicity (Green et al., 1984). After treatment of acetaminophen, hepatocytes of acetaminophen-resistant species such as the rabbit retained higher amounts of glutathione, produced no detectable covalent adducts, and metabolized more extensively to polar metabolites compared to hepatocytes from other species. In contrast, hepatocytes of acetaminophen-susceptible species such as the hamster were depleted of glutathione more rapidly, produced more covalent adducts of acetaminophen, and formed polar metabolites at a slower rate than the rabbit hepatocytes.

Although information on S-methyl reactions in the rabbit is limited, a membrane-bound enzymatic activity has been found in rabbit liver microsomes, which catalyzes the transmethylation from S-adenosylmethionine to a series of C1–C3 alkane thiol (Holloway, 1979). Methane and ethane thiols are known to be endogenous toxins, which may play an important role in the pathogenesis of hepatic coma, and methylation could provide an important pathway for metabolic detoxification through neutralization of the highly reactive sulfhydryl group.

The rabbit appears to have low to moderate sulfate conjugation capability. After administration of dopamine, about 1% of the dose was excreted as sulfate conjugate in the 0–72 hours rabbit urine, whereas about 61%, 5.3%, and 7.0% of the dose were excreted as sulfate conjugates in the dog, rat, and mouse urine, respectively, over the same time periods. Following administration of phenacetin, the sulfate conjugate was a minor metabolite (4%–9%) and N-acetyl-p-aminophenylglucuronide was the major metabolite in the rabbit, whereas in humans, glucuronide and sulfate conjugates were about equally important (36%–42% and 23%–31%, respectively) (Smith and Timbrell, 1974). However, with phenol, the percentage of the total sulfate conjugate excreted in rabbit urine was 54% of the administered dose (Capel et al., 1972). This value is comparable to those in the rat (55%) and mouse (51%) but somewhat lower than that in humans (78%).

The formation of arylsulfo conjugates is known to be rate limited by the endogenous sulfate ion, and thus the extent of conjugation is dose dependent. Liver concentration of adenosine 3′-phosphate-5′-phosphosulfate (PAPS), which is required for sulfation reactions as the sulfate donor, was 32.7 nmol/g of tissue in the rabbit. This concentration was about half the value in the rat, similar to that in the hamster and mouse, but about twofold higher than that in the dog (16.1–17.3 nmol/g). The concentrations of PAPS in the rabbit kidney, lung, and intestine were approximately one-third of the value in the rabbit liver.

The rabbit utilizes the glycine conjugation pathway in the metabolism of many arylacetic acid compounds, such as phenylacetic acid, indoleacetic acid, and phenylcysteine. In contrast, humans did not form any substantial amount of glycine conjugate with these compounds. Interestingly, 1-naphthylacetic acid was conjugated only with glycine in addition to glucuronic acid in the rabbit, whereas 2-naphthylacetic acid formed conjugated metabolites with glycine, glutamine, and taurine as well as glucuronic acid (Emudianughe et al., 1978, 1979).

The drug-metabolizing enzymes in the rabbit are generally induced or inhibited with the known enzyme inducers or inhibitors in the other species, respectively. However, the effects of some inducers and inhibitors have been reported to differ in rabbits from those in the other species. When differential inductive effects in the rabbit liver were studied after treatment with phenobarbital, G-naphtho-flavone (BNF), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and 3-methylcholanthrene (3-MC), metabolism of 2-AAF to the mutagenic metabolite N-hydroxy 2-AAF was increased from 7- to 13-fold after treatment with 3-MC, BNF, or TCDD (Atlas et al., 1975). The N-hydroxylation of 2-AAF increased twofold with phenobarbital in the rabbit but not in the rat, mouse, hamster, and guinea pig. A more detailed study revealed that in the rabbit, a significant portion of the microsomal metabolism of 2-AAF to the mutagenic metabolite was initiated by deacetylation to aminofluorene followed by N-hydroxylation, which was catalyzed by phenobarbital-inducible CYP-450 LM_5 as well as by 3-MC-inducible CYP-450 LM_4.

Acetanilide hydroxylation in the rabbit was induced from 2.4- to 3.9-fold with BNF, TCDD, 3-MC, and phenobarbital (Atlas et al., 1975). However, aryl hydrocarbon hydroxylase activity toward benzo(a)pyrene was increased only by phenobarbital but not by any of the other inducers in this study. Furthermore, Aroclor 1254 and methylcholanthrene, which were potent inducers of N-hydroxylation of 4-aminophenyl in the rat and guinea pig, were not inducers in the rabbit (McMahon et al., 1980). Interestingly, TCDD stimulated the demethylase activity for p-nitroanisole in the rabbit after 3 days of treatment, but the effect was lost after 6 days.

CYP-450 LM_5, which is found in rabbit liver and extrahepatic tissues, does not appear to be similar in all properties to any CYP-450 isozymes isolated from other species. Although phenobarbital induces the synthesis of both isozymes LM_5 and LM_2 in rabbit liver, the relationship between these isozymes is not comparable to that between $P-450_b$ and $P-450_e$, highly related isozymes that are induced by phenobarbital in rat liver.

Vanderslice et al. (1987) examined the presence of homologs of rabbit CYP-450 LM_5 in pulmonary and hepatic preparations from the rat, mouse, hamster, guinea pig, and monkey. Homologs of isozyme LM_5 were detected in pulmonary preparations from all five species. However, only hepatic preparations from the hamster, in addition to those from the rabbit, contained detectable levels of this isozyme. Although LM_5 isozyme in rabbit liver was induced by phenobarbital, treatment of other animals with phenobarbital did not increase the hepatic or pulmonary content of isozyme LM_5 homologs or the amount of 2-aminofluorene metabolism that was inhibited by antibodies to isozyme LM_5. Subchronic treatment of phenobarbital induced renal cortical microsomal monooxygenase and aryl hydrocarbon hydroxylase and CYP-450 content in rabbits but did not induce these renal enzyme systems in the rat (Kuo et al., 1982).

In addition to cytochrome LM_5 isozyme, rabbit pulmonary CYP-450 comprises two other isozymes LM_2 and LM_6. In the untreated rabbit lung, cytochrome LM_2 and LM_5 isozymes are present in approximately equal proportions. However, the isozyme LM_6 has been identified only after treatment with TCDD. In the liver, the isozymes LM_2 and LM_5 make up a small fraction of the total complement, which comprises at least nine forms. Phenobarbital, an inducer of forms 2 and 5 in the liver, has no inductive effect in the lung. Induction of form 4 by aromatic hydrocarbons occurs in the liver but not in the lung, except perhaps in the neonate. Pulmonary concentrations of isozyme 2 were decreased to trace levels by the administration of Aroclor 1260 (Ueng et al., 1980; Ueng and Alvares, 1981; Serabjit-Singh et al., 1983). The pulmonary content of isozyme 6 was also decreased twofold by treatment with phenobarbital but increased 5- and 10-fold by administration of Aroclor 1260 and TCDD, respectively.

It has been reported that metabolism of stereoisomers was selectively induced by pretreatment with different inducing agents. For example, when the stereoselective glucuronidation of oxazepam was studied using rabbit liver microsomes with a series of inducing agents, the ratio of diastereomeric products produced varied dramatically relative to noninduced animals (Yost and Finley, 1985). The R/S enantiomer ratio for noninduced rabbits was 0.76, but this was reversed to 1.41 after

treatment with β-naphthoflavone. These data clearly suggest that various forms of glucuronyl transferase with different stereoselectivities were present in rabbit liver.

α-Naphthoflavone has been reported to be a potent inhibitor of reconstituted rabbit liver P-450 IA2 (LM_4) biphenyl 4-hydroxylation (IC_{50} = 27 nM) and 7-ethoxyresorufin-O-demethylation (IC_{50} = 10 nM) (Johnson et al., 1979). In contrast, P-450 IIB4-dependent activity (rabbit LM_2) was only inhibited to a minor extent at an α-naphthoflavone concentration of 240 nM. Thorgeirsson et al. (1979) also studied the in vitro effect of α-naphthoflavone on four hepatic monooxygenase activities (aryl hydrocarbon hydroxylase, 2-AAF N-hydroxylase, biphenyl 2-hydroxylase, and biphenyl 4-hydroxylase) before and after methylcholanthrene treatment of the rabbit and other laboratory animals (mice, rat, hamster, and guinea pig). In vitro addition of α-naphthoflavone selectively inhibited 2-AAF N-hydroxylase and biphenyl 4-hydroxylase activity in the methylcholanthrene-treated rabbit, which is consistent with the findings of Johnson et al. (1979). However, in vitro addition of α-naphthoflavone enhanced the activities of aryl hydrocarbon hydroxylase and biphenyl 2-hydroxylase in liver microsomes from both control and methylcholanthrene-treated rabbits. The enhancement of monooxygenase activities by the in vitro addition of α-naphthoflavone may be caused by an interaction with the allosteric binding site(s) on the heme proteins. In contrast to the rabbit, in vitro addition of α-naphthoflavone selectively inhibited all four monooxygenase activities from the 3-MC-treated rat, mouse (C57BL/6N strain), and hamster.

Species differences in toxicity of a compound may be due to factors other than differences in its metabolism. Some of these factors include protein binding and biliary excretion in addition to absorption and renal elimination of drugs. When protein binding of some drugs (clofibric acid, etodolac, tolrestat, perricone, benoxaprofen) was examined in the rabbit and other laboratory animals (rat, mouse, dog, rhesus monkey) as well as in humans, the binding in the rabbit appeared to be medium. In general, the binding was highest in human serum and weakest in the mouse. However, when the protein binding of prednisolone was studied in serum from rabbits, dogs, rats, and humans, the binding characteristics of the drug in rabbit serum were most similar to those in human serum (Rocci et al., 1980). A similar phenomenon was observed with a new cephalosporin, CL284635 (Bialer et al., 1986).

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The Ferret

Daniel E. McLain and Shayne Cox Gad

CONTENTS

TOXICOLOGY

Daniel E. McLain

Interest in the ferret as a laboratory animal has grown in direct concordance with the escalating public opposition to the use of "domestic" animals in scientific research and, conversely, with the more recently identified need for a smaller nonrodent species to be used as a surrogate for the canine. To this end, conventional nonrodents such as the canine are being increasingly excluded from the testing of genetically engineered human biologics, for example, because a sufficient quantity of test material simply might not be available. The search for a surrogate nonrodent has, therefore, been focused primarily on smaller contemporary species considered more accommodating to the task at hand. One such small laboratory-adapted carnivore that has found increasing application in this present environment is the ferret.

Scientific Application of the Ferret and the Extent of Its Use in Toxicology

Research with the ferret falls into two main categories: that directed toward gaining basic knowledge applicable to human health and the more recent efforts directed toward ensuring the safety of humans (and their environment) exposed to a multitude of chemical and biological agents. Much of the knowledge gained from the basic biomedical research studies with ferrets has, quite fortunately, found application in studies of the problems related to human toxicity assessment. In this respect, Thornton et al. (1979), Beach (1982), and Hoar (1984) have previously described the applicability of ferrets in the toxicology laboratory, and several symposia devoted to an analysis of uses of the ferret in preclinical safety studies and biomedical research highlighted important contributions (Fox, 1987; Greener, 1987; Haddad and Hoar, 1981). The use of ferrets in such diverse biomedical research areas as cardiology (Breisch, 1980; Marino et al., 1981; Marino and Severdia, 1983), ophthalmology (Braekevelt, 1982; Thorpe and Herbert, 1976; Vinegar et al., 1982, 1985; Wen et al., 1985), virology (Bird et al., 1983; Chevance et al., 1978; Kauffman et al., 1982), bacteriology (Fox et al., 1982, 1983; Koshimizu et al., 1982), toxicology (Brantom et al., 1977; McLain et al., 1987), and developmental toxicology (Beck, 1978; Beck et al., 1978; McLain and Roe, 1983; McLain et al., 1985b) has become frequent and important.

The extent of the research effort with ferrets is further indicated by recent surveys of the scientific literature accessed through the BIOSIS and MEDLINE computerized database search systems, which cover entries from more than 8000 journals in more than 70 different countries. The first selected list of references on the use of ferrets in biomedical research was published by Frederick and Babish (1985) and includes more than 569 entries of papers issued from 1977 to 1984. A later survey published by Clingerman and Fox (1991) contains more than 715 scientific citations dedicated to the use of ferrets as laboratory animals, although some of these are duplicate citations from the Frederick and Babish (1985) survey.

Earlier compendia of similar scientific literature were published by Marshall and Marshall (1973) and Shump et al. (1976), which span the years 1866–1974. As indicated by these literature reviews, the number of scientific publications specifically mentioning the ferret in their title or as a keyword prior to 1974 averaged approximately 5 per year, whereas from 1977 to 1983, the number increased to

approximately 113 per year. Although only 23% of these latter citations were toxicological in content, they can be viewed as an indication of the ferret's increasingly important role in the area of toxicology research.

The increasing number of scientific applications of the ferret and the unprecedented growth of the biotechnology industry correlate highly with commercial sales of the animal over the last 10 years. Therefore, whether used as a surrogate nonrodent for testing the efficacy and safety of limitedly available human biologics or as a simple alternative to more conventional laboratory carnivores, the present usage rate of ferrets suggests that information delineating the species' applications and restrictions in the toxicology laboratory is duly warranted. This chapter, therefore, is an attempt to consolidate current information on the ferret that might be applicable to preclinical safety evaluations. More current information can be found by accessing the National Agricultural Library website (http://www.nal.usda.gov) and searching for "Information Resources on Ferrets, 1991–2002."

History

Taxonomy and Origin

The present-day domesticated ferret, *Mustela putorius furo* (see Table 7.1 for taxonomic classification), is believed to have originated in North Africa more than 5000 years ago (Hagedoorn, 1947; Miller, 1933; Thomson, 1951). Some accounts credit the Egyptians with the domestication of ferrets (ca. 3000 BC) and their subsequent introduction to Europe, Asia, and Great Britain (Owen, 1969; Pyle, 1940). Most historical reviews describe the modern laboratory ferret as descending from the wild European ferret or polecat (*M. putorius* or *M. furo*) or the steppe polecat (*M. eversmanni*), with which they can breed and produce offspring (Fox, 1988; Pocock, 1932). The black-footed ferret (*Mustela nigripes*), an endangered species native to the western United States, is a distinctly different strain and is not used for research purposes (Boggess et al., 1980; Clark, 1978; Moody et al., 1985).

The Mustelidae family of the ferret is considered to be the most primitive living group of terrestrial carnivores, with a total of 70 different species in 25 genera. Genera such as *Lutra* (river otter), *Pteronura* (giant otter), *Aonyx* (small clawed or clawless otter), and *Enhydra* (sea otter) are aquatic, whereas most other members of the Mustelidae family are land dwelling (fissipedia). In the widely distributed Mustilinae subfamily in particular, there are approximately 11 genera and 33 recent species. The more familiar relatives of the ferret include the weasel (ermine, sable), mink, marten, polecat, fisher, skunk, wolverine, otter, and badger. Less commonly known relatives include

Table 7.1   Taxonomic Classification of Ferrets

Kingdom	Animal
Subkingdom	Metazoa
Phylum	Chordata
Subphylum	Vertebrata
Class	Mammalia
Subclass	Eutheria
Order	Carnivora
Suborder	Fissipedia
Family	Mustelidae
Subfamily	Mustelinae
Genus	*Mustela*
Species	*putorius*
Subspecies	*furo*

the zorille and ratel (honey badger). In Europe and Great Britain, the wild ferret is also known as a fitch, fitchew, fitchet, foul marten, or foumart.

Most mustelids have well-developed anal scent glands. The skunks, zorille, marbled polecat, stink badger, and ratel can forcibly eject the vile-smelling liquid as a spray or fluid. In contrast, the laboratory ferret is incapable of projecting its anal gland fluid, which many researchers also rate low on the odoriferous discomfort scale.

Economic Applications

Early Greek Ferreting and Falconry

Early Greek literature indicates that ferrets made their first social impact as hunters of rabbits (Thomson, 1951). Similar to the use of Grisons to flush chinchillas from their burrows, "ferreting" for rabbits consists of muzzling a ferret and placing it into one entrance of a rabbit burrow. The characteristic musk odor of the ferret would cause the burrow's occupants to flee by a rear entrance, whereupon they were duly dispatched by the hunter. The Greeks were probably the first to employ falconry as a method of capture for rabbits flushed from their burrow in this manner. However, many hunters simply held a net or clock sack over the rear entrance to the burrow, hoping that their strength could match the speed and force by which the rabbits exited the hole. Ferreting (or "rabbiting") with firearms is viewed as unsportsmanlike and is prohibited in many parts of the United States.

English Ferret-Legging

The English are credited with popularizing the notable sport of ferret-legging, which has seen a resurgence in recent years. In this contest, a competitor's trousers are tied off at the ankles and a ferret is inserted. After tightening the belt, the contestant proceeds to tolerate the ferret's repeated escape attempts. Katz (1987) described the "ideal" ferret for ferret-legging as "having claws like hypodermic needles and teeth like number 16 carpet tacks." Accordingly, the current record for ferret-legging is 5 hours and 26 min, a mark held by a 72-year-old Yorkshire man.

Use of the Ferret as a Rodent Exterminator

In the early part of the twentieth century, ferrets were popular as rodent exterminators in the United States and England. However, with the advent of chemical rodenticides, this specific application of the species has waned.

Pet Ferrets and Their Legal Restrictions

Currently, there are an estimated 4 million to 5 million pet ferrets in the United States, with approximately 1 million in the state of California alone. Some states such as Georgia, Massachusetts, New Hampshire, and South Carolina, however, have banned the ownership of ferrets completely, and other states such as California, Minnesota, and New York have restricted ownership by requiring licensing, neutering, or leashing. Some localities, such as Carson City, Nevada, have gone as far as to prohibit the sale of ferrets to households that have children under the age of 3 years. These various restrictions have apparently resulted from controversial issues such as whether the ferret can be considered a truly domesticated species, media accounts of instances when young infants have been bitten, and the present lack of a Food and Drug Administration (FDA)-approved rabies vaccine. Proponents of ferrets have, nevertheless, successfully argued their cause in the courts of Alaska, Maine, Pennsylvania, and West Virginia and have had restrictions reversed or overruled in these states. Based on scientific information presented by lobbyists to individual state legislatures, the ferret

is increasingly being categorized as a domesticated animal, thus exempt from control by individual state game commissions. Nevertheless, The Ferret Lovers' Club of Texas (2002) suggests checking local ordinances, or contacting the director of animal control or an equivalent authority to learn about the status of ferret ownership or use (in a given geographical area), as the animals might be prohibited by city or state ordinance or regulated by policy. With respect to domestication, a recent trade journal survey indicated that ferret bites are much less frequent (and severe) than dog bites when expressed relative to species population size, and laboratory researchers have found the ferret to be accommodatingly docile and predictable. On the contrary, Applegate and Walhout (1998) caution that although the ferret is becoming an increasingly popular pet, the dangers of ferret ownership remain unrecognized by physicians and the general public. They described three incidents of ferret attacks in a 3-month period, concluding that the risk of attack is greatest for infants and small children and that wounds caused by ferret attacks must be evaluated for injury, infection, and rabies prophylaxis.

Early Biomedical Research Studies with the Ferret

Pioneering biomedical research studies utilized the ferret in experiments concerned with the pathogenesis of human influenza virus (Moody et al., 1985; Pyle, 1940; Smith et al., 1933), and because of its sensitive pulmonary vasculature, the species continues to be viewed as a valuable model for investigating pulmonary hypotension in humans (Andrews, 1988; Vinegar et al., 1982). In a recent example of the ferret's utility in influenza virus research, de Jong et al. (2000) were able to demonstrate a mismatch between the 1997/1998 influenza vaccine and the major epidemic A(H3N2) virus strain as the cause of an inadequate vaccine-induced antibody response to this strain in the elderly. In that respect, the success of influenza vaccination depends largely on the antigenic match between the influenza vaccine strains and the virus strains actually circulating during the season. In the past, this match has proved to be satisfactory in most seasons. In the 1997/1998 season, however, de Jong and coworkers noted that hemagglutination inhibition (HI) assays with ferret antisera indicated a considerable mismatch between the H3N2 vaccine component and the most prevalent epidemic influenza A(H3N2) virus. The results from their antigenic analyses using pre- and postvaccination serum samples from volunteers of various ages, including residents of nursing homes who were more than 60 years of age, were in good agreement with the results obtained with ferret antisera. Homologous serum antibody responses to the H3N2 vaccine component as well as the cross-reactivity of the induced antibodies to the epidemic H3N2 strain declined with increasing age of the vaccines. As a consequence of these two effects, 84% of the vaccines over 75 years of age did not develop HI antibody titers ≥ 40 against the major H3N2 virus variant of 1997/1998, suggesting that they were not protected against infection with this virus variant. The authors concluded that these findings support the current policy of the World Health Organization (WHO), which is to base worldwide influenza virus surveillance on results predominantly obtained by antigenic analyses of influenza virus isolates with ferret antisera in HI tests. If an antigenic mismatch is observed, the protective efficacy of the vaccine, especially for the elderly, might be insufficient.

In the more contemporary area of DNA vaccine research, scientists at Merck Laboratories (Donnelly et al., 1995) used ferrets to demonstrate that DNA vaccines were more effective, particularly against different strains of virus, than inactivated virus or subvirion vaccines. They reported a comparison of DNA vaccines, using contemporary human strains of virus, and clinically licensed (inactivated virus or subvirion) vaccines in preclinical animal models, to better predict their efficacy in humans. Influenza DNA vaccines elicited antibodies in both nonhuman primates and ferrets and protected ferrets against challenge with an antigenically distinct epidemic human influenza virus more effectively than the contemporary clinically licensed vaccine. Along this same line, Webster et al. (1994) demonstrated that immunization of ferrets with a plasmid DNA expressing influenza virus hemagglutinin (pCMV/H1 DNA) provided complete

protection from challenge with the homologous A/PR/8/34 (H1N1) influenza virus. They provided evidence that delivery of DNA-coated gold beads by gene gun to the epidermis was much more efficient than intramuscular delivery of DNA in aqueous solution. They also provided evidence that the antibody response induced by DNA delivered by gene gun was more cross-reactive than DNA delivered in aqueous solution or after natural infection and concluded that this novel approach to vaccination against influenza might afford broader protection against antigenic drift than that provided by natural infection. Interest in the ferret as an animal model for influenza infection and the use of plasmid DNA vaccines delivered to the human epidermis have increased considerably since these early reports were published. Ferrets have also been used quite successfully in assessments of reproductive toxicity (Beck et al., 1978; Gulamhusein et al., 1980; McLain and Roe, 1983), and a Stanford University symposium (Haddad and Hoar, 1981) and several review articles (Beck, 1975; Gulamhusein and Felix, 1977; Haddad and Rabe, 1980) have highlighted the species' many advantages and contributions to this specific scientific discipline. Beck (1975, 1981) and Hoar (1984), for example, contrasted human, rat, and ferret placentas and concluded that the latter was most similar in structure and function to the human. Literature reports continue to demonstrate similar reproductive responses in humans and ferrets (Beck et al., 1978; Collie et al., 1978; Elizan et al., 1969; Gulamhusein et al., 1980; McLain and Roe, 1983).

Husbandry

Specific husbandry practices for the ferret are summarized in Table 7.2. Details of selected parameters are outlined in the following sections.

Table 7.2 Recommended Husbandry Practices for Ferrets Maintained under Controlled Environment Conditions

Parameter	Units	Value
Cage dimensions (L × W × H)		
Breeding (2 adults)	cm	60 × 60 × 40
Growing (1–8 kits)	cm	40 × 40 × 30
Experimental	cm	Varies
Cage bar spacing	cm	<2.5
Area temperature	°C	15 ± 5
Area humidity	%	50 ± 20
Room air changes	No./h	10–15
Lighting (incandescent or fluorescent)		
Sexually inactive	h/day	8 ± 2
Sexually active	h/day	14 ± 2
Nest material (other than 3/8 in. grid flooring)		
All ages	—	Shavings
Pregnant females	—	Nest box
Food intake (not accounting for spillage)		
Energy (maint.)	kcal/kg/day	200–300
Dry matter	g/day	50–70
Solid food, age at	Weeks	1–2
Diet peculiarities		
Growing	—	Ad libitum
Breeding, lactating	—	Ad libitum
Adult	Low body fat	Limit feed
Water requirements	mL/24 h	75–100 or ad libitum

Caging and Bedding

The staff veterinarian or designee should be responsible for determining suitable housing for newly arriving ferrets. In addition, rooms in which ferrets are housed should be of adequate space, clean, ventilated, and environmentally controlled in accordance with government regulations and guidelines established for the care and use of laboratory animals and in accordance with applicable facility standard procedures. Before modifying details of an established animal-care program, the staff veterinarian (or designee) should notify the research scientist whose animals are affected by the proposed change.

The recognition that ferrets adapt readily to the various types of caging routinely found in the small animal vivarium has undoubtedly contributed to the success of the species in the biomedical research laboratory. Stainless steel rabbit cages (2′ L × 2′ W × 1′ 3″ H), for example, can house one or two ferrets quite comfortably. Cat cages (2′ L × 2′ W × 2′ H) are quite adequate when group housing is preferred. The most recent issue of the National Institutes of Health (National Research Council, 1985) *Guide for the Care and Use of Laboratory Animals* did not specify standards for the size of ferret caging, but it is likely that either of the dimensions just discussed will more than satisfy any potentially forthcoming criteria. Both cage types described are low stress, providing for sufficient lateral (horizontal) and vertical (rearing) motion. In addition, they exceed the dimensions specified by Wilson and O'Donoghue (1982) and by the Royal Society of England (Andrews and Illman, 1987). Wilson and O'Donoghue (1982) have described a mobile rack of cages for ferrets.

Group housing and opportunity for interaction suitable to ferrets should be provided whenever possible, but it should also be recognized that group housing of mature adults is frequently impractical (see the following discussion). Therefore, wherever possible, caging should be provided that permits easy viewing of neighboring cages and their occupants.

Limitations of Cage Bar Dimensions

A variety of cage dimensions might be satisfactory for housing ferrets, as long as the interior dimensions provide for uninhibited movement and the cage bar spacing of the unit is less than 2.5 cm, or modified appropriately. With respect to cage height, behavioral observations indicate a natural rearing motion of this species (similar to cats and dogs), which is probably related to a strong dependency on smell when hunting and the need to extend their head above tall grasses to examine the upper air currents. Observations have also indicated that ferrets with body weights less than 700 g will escape through any cage bars spaced 2.5 cm or more apart as well as through the popular J-type gravity feeder. Therefore, containment should be assured by employing commercially available stainless steel grid door inserts (Lab Products, Inc., Maywood, New Jersey, ¼ in. grid) and by locating feed dishes inside the cage bars. It is also possible to insert an appropriately shaped section of stainless steel grid (spacing approximately ¾″ H × 1½″ L) inside the J-type gravity feeder, which prevents escape and reduces the digging and scattering of feed (see the following discussion).

Excretory Habits of Caged Ferrets

Ferrets are accommodating in that they characteristically deposit their wastes in distinct corners of the cage. Thus, cage floor grids should be large enough to allow excreta to fall through to the catch pans below, yet small enough so as not to cause the animal discomfort when walking or to allow escape. Commercially available washdown rabbit caging with $\frac{3}{8}$ in. grid flooring fits these criteria and requires little maintenance time for hygiene control. Alternatively, direct-contact bedding material (wood chips, paper strips, etc.) can be used with similar success, with soiled corner material removed daily and complete changes occurring as needed. For short-term housing or transportation, some investigators have employed the large polycarbonate rat breeding cage (9″ H × 9″ W × 16″ L),

covering the bottom with wood chips to facilitate cleaning. As indicated, however, these units should not be used for any extended housing period because of their small size. Moreover, the units should be dedicated to ferrets because they will tend to retain the musk odor, causing considerable consternation for the next small rodent inhabitant.

Nest Boxes for Pregnant Ferrets

A nest box (approximately 6″ H × 6″ W × 12″ L) constructed of either metal or plastic, partially filled with wood chip or paper strip bedding material and containing a 2–3 in. diameter entrance hole, is a valuable cage addition for the pregnant jill and for increasing the comfort of animals subjected to chronic treatment protocols. Nontransparent nest boxes such as metal, however, are not conducive to recording detailed daily clinical observations because the animals will tend to spend a disproportionate amount of time inside them. The smaller-sized polycarbonate rat and mouse cages have been employed with greatest success. Entrance holes should be cut approximately 2 in. above the floor level, which prevents the newborn kits from falling out while permitting easy access for the jill.

Individual Caging Requirements of Adult Ferrets

Littermates of both sexes can be group housed until sexual play or aggressive behavior becomes too violent. Thereafter, individualized caging is required, especially during periods of peak sexual activity and pregnancy, or when suppression of estrus is desired. Sexually active male ferrets, and to a lesser extent females, will fight when housed with others of the same sex. Similarly, although the species is not prone to cannibalism, jills that have just whelped should be allowed to recover and nurse their litter of kits undisturbed. Segregating by sex and by room will generally help to suppress the incidence and onset of estrus in females. Conversely, desegregation might promote the onset of sexual activity, and the sexual play of females might elicit ovulation or a pseudopregnancy.

For breeding purposes, it is recommended that receptive females be transferred to the sexually active male's home cage. When coitus is completed, the female should be returned to her own cage.

Reproductive sterilization apparently reduces aggressive behavior sufficiently such that animals can be group housed. However, a pecking order or individual domination might still become manifest.

Lighting

Similar to the effects of altered photoperiodism documented for mink, the light–dark cycle employed in the ferret vivarium can be manipulated to control the breeding cycle. Ferrets are seasonal breeders and will become conditioned for breeding with exposure to longer day lengths or periods of artificial illumination. To prevent the onset of sexual activity (or first estrus) in younger animals arriving at the vivarium during the natural breeding season (January–March), or to maintain a colony of stock animals in a sexually inactive state, lighting is optimally set between 6:18 and 10:14 light/dark to simulate the early winter (nonbreeding) months (Moody et al., 1985; Ryland and Gorham, 1978). Conversely, increasing the period of illumination to between 12 and 16 hours might stimulate sexual activity in this species (Donovan, 1967). Detailed discussions of the effects of day length on the coat-shedding cycles, body weight, and reproduction of the ferret have been published by Harvey and MacFarlane (1958) and Donovan (1966, 1967). Earlier but equally informative studies were reported by Bissonnette (1932), Hart (1951), and Hammond (1952).

It is noteworthy that loss of hair, which can proceed to the development of alopecia, is observed in ferrets (especially marked in estrus females) subjected to long exposure of 16 hours of light daily. Conversely, marked growth of hair takes place under short-day conditions. Because marked hair growth can help to confirm that animals are being maintained in a sexually inactive state, as opposed to pregnancy or pseudopregnancy (marked by hair loss), the condition of the coat should be monitored

and included as part of the clinical observations protocol. A classic study of the molting and fur growth pattern in the adult mink, which applies to the ferret, was published by Bassett and Llewellyn (1949).

Temperature and Humidity

Because of their thick fur, ferrets will tolerate low environmental temperatures, and, in fact, many commercial producers began their own business by group housing their sexually inactive animals in outdoor sheds throughout the year. Freezing winter temperatures were evident when the high-moisture diets fed to these animals froze on the cage floor pallets. Prolonged cold spells simply caused these animals to mold together into a single, continuous ball of fur buried deep beneath the straw bedding. This natural tolerance for cold temperature can be extrapolated to the vivarium.

For maximum thriftiness of mature, individually housed animals, environmentally controlled rooms should maintain an optimal temperature of $15°C \pm 5°C$. Thickly furred adults will benefit from the lower end of this temperature range. Conversely, lactating jills and their unweaned litters might require the slightly warmer temperatures at the upper end of this range.

As with most other species, high relative humidity is apparently not an important factor in ferret performance, except when accompanied by prolonged elevation in temperature. The recommended relative humidity for the vivarium is $50\% \pm 20\%$.

Clinical Signs of Heat Stress

Ferrets are particularly vulnerable to heat stress, and special care is necessary when moving them from one area to another during hot weather. Adult ferrets and breeding stock are especially sensitive owing to their subcutaneous fat cover and their inability to dissipate heat by sweating. Clinical signs of hyperthermia include panting and mouth breathing, prostration followed by eventual flaccidity, and emesis. When a tiered caging system is used, particular attention should be paid to animals housed in the tier closest to the ceiling, where the warmer air can concentrate.

Room Air Changes

Regardless of species, most modern vivariums are designed to maintain a range of 10–15 complete exchanges of room air (nonrecirculated) per hour. Ferrets do well under these conditions, but because of their musky odor and susceptibility to respiratory pathogens, the higher end of the range is frequently indicated. In the authors' experience, environmentally controlled animal rooms measuring $14' \times 22'$, employing nine racks of nine cages per rack of washdown caging (cleaned with water once daily), nonabsorbent wall and floor surfaces, and 15 air exchanges per hour will not retain an odor even when ferrets are housed two per cage at maximum room density.

Diet and Water

Natural Ingredient and Purified Diet Formulations

A recent survey (McLain et al., 1985b) of a large commercial ferret colony (Marshall Farms, North Rose, New York) detailed a successful ingredient diet for ferrets (Table 7.3). Reproduction data reported in this survey demonstrated that primiparous females fed this natural ingredient diet whelped an average of 10.3 ± 0.2 kits per litter (range = 1–18), successfully weaned 80% of these, and frequently produced three to four litters per year. Subsequently, the Marshall Farms natural ingredient ferret diet was analyzed and reproduced as a purified formulation (McLain and Roe, 1983; McLain et al., 1988). Moreover, when prepared with animal fat and $\geq 50\%$ moisture (using 2% agar), adequate weight gain is demonstrated for animals consuming this purified formulation.

Table 7.3 Composition of a Successful Natural Ingredient Ferret Diet

Ingredient	Approx. Wt. per Batch[a]
Wayne dog food cereal	100
Agway dog food cereal	50
Beef tripe	100
Beef lung	50
Beef liver	10
Fortified cod liver oil	1
Water	(varies)

Source: Courtesy of Gilman Marshall, Marshall Farms, North Rose, NY.
[a] Total batch weight is approximately 1000 lb. Dry ingredients are added to a mechanical mixer from bags or cans; water is added until desired consistency is achieved.

Commercial Diets

Two large commercial producers of animal feed presently market least-cost formulation ferret diets that also promote adequate weight gain. Ralston Purina (St. Louis, Missouri) offers the Purina 5280 ferret chow, which is pelleted and available as a certified ration. Agway, Inc. (Ithaca, New York) also offers a pelleted ration that closely parallels the nutrient composition of the Marshall Farms natural ingredient diet.

Consumption patterns and acclimation of ferrets to commercial rations. For maintenance of body weight, ferrets will require between 200 and 300 kcal/kg body weight daily. This translates to between 50 and 70 g/day of the pelleted commercial rations described previously, not accounting for spillage (McLain and Roe, 1983; McLain et al., 1988). Bleavins and Aulerich (1981) reported food consumption to average 43 g/kg body weight in the ferret, and mean food passage time (as measured by a dye marker in the feed) was reported to average approximately 3 hours, indicating a relatively short digestive tract.

The study of food habits of ferrets living in the wild has resulted in several interesting and informative behavioral publications (Kaufman, 1980; Roser and Lavers, 1976). However, nothing has been published that will prepare the naive investigator for an unacclimated ferret's first encounter with a pelleted diet offered in a stainless steel or glass bowl. Initially, new arrivals will appear to immensely enjoy the sound of digging in metal or glass food dishes and subsequently spilling their daily allotment of food. To cure this habit, replenish the food only during designated feeding times. By approximately the third day, the animal will have lost body weight and be sufficiently hungry so that food pellets moistened slightly with water will be ravenously consumed. Gradually decrease the amount of water added to the pellets until dry food is consumed exclusively. This process might have to be repeated. Fortunately, the burden of pelleted diet acclimation has been assumed by the more conscientious animal vendors.

On rare occasions, an individual animal might appear to have chosen starvation rather than consuming a dry food ration. In these cases, canned (moist) cat or dog food or fresh organ meats might have to be fed to ensure survival. Although animals receiving these diets and special considerations might have to be excluded from controlled protocol studies, they can still be used subsequently for technical training purposes or exploratory protocols.

Automatic Watering Systems

Many commercial ferret producers use automatic watering systems in their facilities. Consequently, ferrets will readily adapt to the demand-controlled automatic systems present in the newer caging units or to water bottles attached to the cage. As with other species, the valves of automatic watering systems must be checked routinely to ensure that they are functional, and water bottles should be changed daily.

Acclimation and Quarantine Procedures

Special Attention for New Arrivals to the Vivarium

It would be naive to assume that newly arriving ferrets will tolerate the type of handling that is afforded the more "conditioned" animals of this or any other species. In reality, ferrets arriving from most commercial breeders will have been selectively bred for an even disposition, but memories of pecking order and perhaps competition for food and water during shipping might still be fresh in their minds. Many of the new animals will be confused and frightened by the unfamiliar sights and sounds experienced during shipping. A small percentage of ferrets might, in fact, have become overly stressed and understandably resist (e.g., hiss, scream, back away from the hand) any initial attempts to handle them. To reduce the risk of injury to both animal and technician, therefore, shipping crates containing newly arriving animals should be unpacked cautiously (preferably away from bright light or loud noise) and animals offered fresh food and water and dim lighting as soon as possible. Additionally, a qualified animal health technician or laboratory veterinarian should be available to assist in the identification and treatment of any animals that have succumbed to heat or shipping stress. Ferrets will generally respond rapidly to this initial attention paid to them by allowing handling and petting within only hours of arrival. Slower responding animals will require more patience, but will usually assume a more even disposition with additional efforts during acclimation.

Initial Physical Examination Parameters and the Observations
Recorded during the Period of Quarantine

All animals should be subjected to a complete physical examination within 1–2 days of arrival. Examination should include inspection for disorders of the skin and fur (ectoparasites), nose and throat, eyes and ears, and obvious physical impairments that would preclude the use of the animals in scientific research, fecal and urine screening (see later section on urinalysis), and the presence and condition of vendor-applied ear tags. The quarantine period for ferrets should be long enough to allow an evaluation of daily clinical observations, body weight change, and food and water consumption patterns for their individual contribution to the animals' physical health. Routine clinical observations and body weight changes by themselves, when recorded over a 1–2-week period, will usually enable the identification of animals infected with either respiratory or intestinal pathogens. Optimum prophylaxis requires that diseased animals be culled or isolated from healthy animals and administered appropriate professional health care.

Depending on the scientific application, blood samples might or might not be collected from animals during the quarantine period (see later section on blood sampling techniques).

A typical animal history chart for use during the quarantine periods of ferrets is shown in Figure 7.1.

Veterinary Procedures and Common Diseases

General veterinary care of the ferret is similar in many aspects to that provided for the feline and canine species. Approximate drug dosages, when required for specific veterinary procedures or prophylaxis, are listed in Tables 7.4 and 7.5. Confirmation of these dosages is recommended for each laboratory before routine use.

Anesthetics and Drug Dosages

Anesthesia, sedation, or tranquilization, although not mandatory, will frequently facilitate initial, detailed physical examinations of the ferret and significantly reduce the potential for inadvertent injury to the examiner or animal. In addition, mild tranquilization will also facilitate examination of

Figure 7.1 Quarantine information chart for ferrets. Additional physical examination data should be added as required by each facility.

the eyes and have an indirect calming effect on the ophthalmologist as well. The following sections, therefore, provide general information concerning recommended agents, routes of administration, and doses of anesthetics, tranquilizers, and other various drugs frequently used in the ferret. These guidelines are intended to comply with appropriate sections of the Animal Welfare Act.

General Anesthetics

General anesthetics used successfully in ferrets include those drugs that produce controllable and reversible loss of consciousness with analgesia. The same general anesthesia principles apply to ferrets as would be practiced with most other species. For example, when possible, withhold food from ferrets prior to anesthetic administration. In addition, give a general physical examination immediately prior to treatment to evaluate the general condition of the animals in terms of anesthetic risk. Once anesthesia is induced, monitor the animals every 15 min for heart and respiration rate, mucous membrane color, capillary refill time, and plane of anesthesia. During recovery from anesthetics, isolate the animal, place it in sternal recumbency until swallowing and jaw reflex return, and provide for maintenance of body temperature. Ferrets should be turned to the opposite side every 30 min during prolonged recovery to prevent hypostatic congestion of the lungs.

Table 7.4   Guidelines for Sedative, Preanesthetic, and Anesthetic Dosages in the Ferret[a]

Sedative	
ACE	0.2–0.5 mg/kg im, sc
KET	10–20 mg/kg, im
Xylazine (XYL)	1.0 mg/kg im, sc
Diazepam (DZP)	1.0–2.0 mg/kg, im
Preanesthetics	
ACE	0.01–0.25 mg/kg im, sc
Atropine	0.05 mg/kg im, sc
Anesthetics	
KET	30–60 mg/kg, im
KET + ACE	KET: 20–35 mg/kg
(100:1 mixture)	ACE: 0.2–0.35 mg/kg, im
KET + valium	KET: 25 mg/kg
(10:1 mixture)	VAL: 2.5 mg/kg, im
XYL (followed by KET)	XYL: 2–3 mg/kg, sc
	KET: 20–30 mg/kg, im
DZP (followed by KET)	DZP: 2.0–3.5 mg/kg, sc
	KET: 20–35 mg/kg, im
Pentobarbital	25–35 mg/kg, ip

[a] Dosages should be confirmed in each laboratory before application.

Table 7.5   Guidelines for Antibiotic, Hormone, and Steroid Dosages in the Ferret[a]

Antibiotics	
Albon (oral, inject)	Feline dose
Albon (coccidian Rx)	Feline dose
Amoxicillin (oral)	25–35 mg/kg bid
Ampicillin	10 mg/kg bid sc
Gentamicin	5 mg/kg im
Griseofulvin tabs	25 mg/kg
Ivermectin	1 mg/kg
Neomycin	10–20 mg/kg
Penicillin (Flo-Cillin)	Feline dose
Piperazine salts	Feline dose
Tetracycline (oral)	25 mg/kg bid
Hormones and steroids	
Dexamethasone	0.25 mg/kg
GnRH	20 μg
HCG	100 IU im
Insulin	1–2 units/kg
Oxytocin	0.2–3 U/kg sc, im
Prednisone (oral)	0.5–2 mg/kg
	Feline schedule

[a] Dosages should be confirmed in each laboratory before application.

The various ketamine (KET) mixtures listed in Table 7.4 are apparently the most frequently used anesthetics for minor surgical and noninvasive procedures, with a 25–35 mg/kg dosage inducing a desirable limpness and flaccidity to the animal body for approximately 30–45 min. Limited observations by one of the authors suggest that the albino strain of the ferret might be slightly more sensitive to KET–acepromazine (ACE) than the more common sable strain. Several informative

discussions of ferret tranquilization, preanesthesia, and anesthesia have been published (Fox, 1988; Moody et al., 1985; Moreland and Glaser, 1985). Unfortunately, very little discussion is afforded to KET–ACE, a mixture with which one of us has had great success.

Preanesthetics. Preanesthetics include those drugs that facilitate effective anesthesia in the ferret. Atropine sulfate and ACE (Table 7.4) are used effectively and are indicated to reduce excessive upper respiratory secretions associated with some anesthetics and to decrease bradycardia (vagal effects). Atropine sulfate, for example, should be administered approximately 15–30 min prior to anesthesia and has a duration of action of approximately 15–30 min. It could cause dilation of the pupils and decreased lacrimation; therefore, use of ophthalmic ointments might be indicated.

Tranquilizers and sedatives. These agents are frequently used to facilitate restraint for nonpainful procedures such as bandaging, radiology, parenteral injections, venipuncture, or examination (including ophthalmic), to decrease excitement in nervous or frightened animals, and, as preanesthetics, to decrease the dose of anesthetic required. Note that these agents are not analgesic or anesthetic in nature and should not be used as pain relievers.

The tranquilizing effect of ACE (Table 7.4) can last several hours in the ferret. Moreover, when combined with KET, it is excellent for analgesia, sedation, and minor surgical procedures. However, because it can cause peripheral vasodilation, thermoregulation should be maintained.

Prophylaxis and Vaccination Schedules

Most commercial suppliers of ferrets routinely vaccinate kits against canine distemper virus (CDV) at approximately 6–8 weeks of age (2 weeks earlier if the jill has not been recently vaccinated), with boosters administered approximately 4 weeks later and every 3 years thereafter. A modified-live distemper virus of chick embryo tissue culture origin has been used successfully, as has the modified-live virus of canine origin. Killed vaccines might be ineffective (Ott and Svehag, 1959), and unattenuated viral vaccines derived from ferret cell culture might be too virulent. Commercial breeders routinely vaccinate pregnant ferrets without harm to the fetus (Hagen et al., 1970). Unvaccinated ferrets exposed to the CDV for only 15 min will acquire the disease, and close to 100% of these animals will die. Because there is no cure for the disease once it is acquired, it is strongly recommended that the initial vaccination and subsequent booster be a prerequisite of animal purchase. A detailed description of the disease in ferrets was published by Ryland and Gorham (1978).

The incidence of rabies in domestic ferrets is very slight. The Centers for Disease Control (CDC) in Atlanta, Georgia, reports only five cases in the United States over a period from 1958 to 1983, and no recent cases have been confirmed. The U.S. FDA has not yet approved a rabies vaccine, but the Canadian Council on Animal Care clearly states in its Guide to the Care and Use of Experimental Animals (Vol. 2) that inactive ("killed") rabies vaccine is effective for ferrets. More recently, according to McBride (1989), Norden Laboratories of Lincoln, Nebraska, has finished preliminary testing on an inactivated ferret rabies vaccine and has begun the challenge test. A ferret rabies vaccine was approved in 1992. Testing has shown a slight difference in serological response from males to females, which could mean ferrets might require a two-dose initial vaccination (recommended vaccination age will be 12 weeks), with boosters (dosage = 1 cc) given every 12 months thereafter.

Surgical Sterilization Procedures and Anal Musk Gland Removal

Reproductive sterilization of laboratory ferrets is accomplished using surgical procedures and conditions typically described for the feline and canine species (Randolph, 1986). Moreover, removal of the ferret's anal musk glands is similar to that documented for other mustelids (Creed and

Kainer, 1981). These procedures (spaying, castration, and descenting) are typically performed by vendors (on request) on animals as young as 8 weeks of age, apparently with minimal blood loss or tissue trauma when the proper anesthesia and surgical techniques are employed. When allowed by scientific protocol, these procedures are recommended for the chronic (repeated), restrained, intravenous (IV, caudal vein) treatment of the stronger smelling and more aggressive male ferrets especially and to preclude estrous cycling of females.

Most Frequently Observed Diseases of Ferrets Maintained under Controlled Laboratory Conditions

Although they are probably 10 breeding years behind the current virus-free and specific pathogen–free (SPF) rats, laboratory ferrets maintained under controlled conditions manifest a relatively low incidence of debilitating diseases. On the contrary, very few diseases with unique clinical signs that would enable early detection for prophylaxis have been documented in ferrets. Moreover, underlying pathological conditions appear to be rarely evidenced by more than a reduction in body weight and a reduction of appetite and food intake. Literature reviews of the various diseases observed in this species have been published by Ryland and Gorham (1978) as well as the very extensive review by Fox (1988). A more recent review has been published by Rosenthal (1994).

Influenza and Pneumonitis

As demonstrated by earlier classic studies (Bell and Dudgeon, 1948; Fisher and Scott, 1944; Smith et al., 1933; Smith and Stuart-Harris, 1936), the human influenza virus can be readily transmitted from human to ferret, ferret to ferret, and ferret to human by way of aerosolized droplets containing infective viral particles. Infection is characterized by lethargy, elevated rectal temperature (40°C–41°C, lasting 1 day and then returning on the third day), and bouts of sneezing accompanied by a mucoserous nasal discharge. Some animals might also manifest a conjunctivitis or otitis. Clinical signs can persist for up to 2 weeks, with resistance to the same strain of influenza virus demonstrated on subsequent exposure. Congestion might be relieved by antihistamines suitable for other species.

Preventive measures include the wearing of face masks by personnel exposed to outbreaks of the viral infection and the isolation of infected animals. Limited prophylaxis has been reported with 6 mg/kg bid aerosolized amantadine hydrochloride (Cochran et al., 1965; Cusumano et al., 1965; Fenton et al., 1977) and to a lesser extent with 100 mg/kg ribavirin (Fenton and Potter, 1977). An additional influenza-like disease includes the respiratory syncytial virus (RSV) infection of infant ferrets (Prince and Porter, 1976; Tyrrell and Hoorn, 1965). RSV also has significant health consequences in the human infant. In the absence of an adequate small animal model for testing the efficacy of adenovirus-vectored RSV vaccines, Hsu et al. (1994) established a ferret model for this purpose. In their studies, recombinant adenovirus types 4, 5, and 7 expressing the RSV fusion glycoprotein (F), the attachment glycoprotein (G), or both F and G were constructed previously. These recombinants contain a deletion of a large portion of the E3 region of the respective adenovirus vector. In addition, the researchers constructed an Ad7 (E3+) F recombinant virus that contains an intact E3 region to assess whether E3 region functions might enhance vaccine immunogenicity. Evaluation of these viruses in the ferret model demonstrated that Ad4 and Ad5 recombinants, administered intranasally to ferrets, induced stronger seroresponses to RSV than did Ad7 recombinant viruses. Ad7 (E3+) F did not show enhanced immunogenicity relative to E3-deleted recombinant viruses. However, measurement of RSV infectivity in nasal washes, following intranasal RSV challenge, showed that five different vaccination regimens, Ad7F/Ad4F, Ad7G/Ad4G, Ad7FG/Ad4FG, Ad4F/Ad7 (E3+)F, and Ad5F/Ad4F, protected ferrets from RSV infection in a dose-dependent manner.

Bacterial pneumonias affecting ferrets are similar to those seen in other species. The list of reported strains includes *Bordetella* sp. (McLain, 1989) and *Streptococcus* sp. (Andrews et al., 1979a), as well as the various gram-negative bacteria (*E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*).

Interstitial pneumonitis in conjunction with focal mononuclear cell infiltrates can be caused by bacterial, viral, or protozoan infections, including *Pneumocystis carinii*. The latter protozoan parasite is known to inhabit the lungs of various domestic and laboratory animals with compromised immune systems (Farrow et al., 1972; Long et al., 1986; Milder et al., 1980). Recently, Stokes et al. (1987) studied the disease in ferrets by immunosuppressing the animals with cortisone acetate (10–20 mg/kg subcutaneous for 9–10 weeks).

Aleutian Disease

Aleutian disease, a persistent and frequently fatal parvovirus infection first reported to be common in the Aleutian mink (Henson et al., 1961; Obel, 1959), also occurs in the ferret (Kenyon et al., 1966, 1967; Ohshima et al., 1978). However, the strain of parvovirus infecting ferrets, although resulting in similar pathological lesions (e.g., hypergammaglobulinemia and plasma cell dyscrasia), is rarely fatal to them. Reviews have been published for Aleutian disease in mink (Porter et al., 1980) and in ferrets (Porter et al., 1982). Mild to moderate lymphocytic infiltrates of the liver (periportal localization), lung (pneumonitis), and kidney (pyelonephritis/glomerulonephropathy) are the predominant histological finding in ferrets with Aleutian disease. Occasionally, an animal will become anorexic and thin and pass black, tarry feces.

Kenyon et al. (1967) have defined the hypergammaglobulinemia associated with Aleutian disease in ferrets as greater than 20% of total serum protein as γ-globulin. Moreover, Porter et al. (1982) demonstrated that ferrets with the highest Aleutian disease virus antibody titer also had the greatest increase in serum gamma globulin levels and the most severe tissue lesions. Because ferrets infected with Aleutian disease are generally asymptomatic and the corresponding changes associated with the disease have potential for misinterpretation, McLain and Lin (1989) developed a method for predicting the degree of periportal lymphocytic infiltrates from routine blood samples. When both pretreatment and posttreatment blood samples are used in the "liver lymphocyte index" (LvLI) equation, the prediction or correlation was shown to have a 92%–96% success rate.

Proliferative Colitis

Fox et al. (1982) described proliferative colitis in ferrets and addressed the histopathological and bacteriological features of this disease entity, which, according to the authors, is fatal or requires euthanasia in almost all affected animals. The etiological agent is probably a *Campylobacter* sp., with fecal–oral spread and food- and waterborne transmission apparently the principle avenues for infection.

Clinical signs of proliferative colitis include diarrhea, anorexia, and marked weight loss (≥10%–15%/week). Significant elevations in urine pH (≥8.0) and urobilinogen (≥2.0) might occur, compared with normal values of approximately 6.0–6.5 and 0.1, respectively. In the experience of one of the authors, the incidence of the disease has been 3 in approximately 1000 animals (newly arriving only), with mortality occurring in only 1 of these. Antemortem fibrinogen was 442 mg/dL in the single fatality, compared with a normal value of 189 ± 78 mg/dL (95% upper tolerance limit [TL] = 387 mg/dL). It is noteworthy that in the same author's experience, no cases of proliferative colitis have occurred in resident animals released from quarantine.

Treatment for proliferative colitis should be instituted immediately on noting diarrhea and anorexia. Amoxicillin (oral suspension, 250 mg/5 cc), Pepto Bismol, and water should be given orally twice a day for 5–7 days in a dosage containing 1 cc amoxicillin, 1 cc Pepto Bismol, and 1 cc water.

Canine Distemper Virus

Mortality in ferrets infected with pathogenic CDV approaches 100%. CDV was previously considered to have a host range restricted to the canid family. In 1994, the virus was associated with sporadic outbreaks of distemper in captive felids. However, after severe mortality occurred in the Serengeti lions (*Panthera leo*), attention focused on the pathogenesis of the virus and a concerted effort was made to identify the virus as CDV or a closely related feline morbillivirus. Evermann et al. (2001) designed a study to explore the susceptibility of ferrets to challenge with two morbilliviruses isolated from lions and the protective effects of a modified-live mink distemper vaccine. Two strains of lion morbillivirus were used as a challenge, A92-27/20 (California lion isolate) and A94-11/13 (Serengeti lion isolate). The two strains of lion morbillivirus were antigenically related to CDV (Rockborn strain), and ferrets were susceptible to both of the viruses when inoculated intraperitoneally. The inoculated ferrets were anorectic at 5–6 days postinoculation (PI), exhibited oculonasal discharge at 9–12 days PI, and became moribund at 12–22 days PI. Severe bilateral conjunctivitis was the typical clinical sign. Inclusion bodies characteristic of morbillivirus (eosinophilic, intranuclear, and intracytoplasmic) were distributed in many epithelial cells, including those of the skin, conjunctiva, gallbladder, liver, pancreas, stomach, trachea, lung, urinary bladder, and kidney. Virus was reisolated from selected lung tissues collected at necropsy and identified by CDV-specific immunofluorescence. Ferrets vaccinated with the mink distemper vaccine (Onderstepoort strain) were protected from challenge with the two lion strains, adding further support to the premise that the viruses are closely related to CDV.

Bone Marrow Hypoplasia and Estrogen-Induced Anemia

Aplastic anemia has been associated with prolonged estrus in the female ferret (Kociba and Caputo, 1981). Similarly, bone marrow hypoplasia in estrous ferrets has been attributed to prolonged exposure to estrogens. Because unbred female ferrets will remain in estrus for the duration of the normal breeding season (March–August), the potential for this pathological condition to occur would seem high.

Clinical signs of bone marrow hypoplasia include cutaneous petechiae and ecchymoses, GI hemorrhages, systemic bacterial infections, and pale mucous membranes (Kociba and Caputo, 1981). Hematological findings include initial thrombocytes and leukocytosis followed by thrombocytopenia, leukopenia, and anemia. Decreased platelets (<50,000/μl), hemorrhagic anemia, and death can occur in 40%–50% of affected animals. Histopathological findings include bone marrow hypoplasia affecting all cell lines and decreased splenic extramedullary hematopoiesis (Sherrill and Gorham, 1985). Prevention of the condition requires sterilization or breeding of estrous females, a controlled lighting regimen to preclude estrus cycling, or an intramuscular injection of human chronic gonadotropin (HCG, 100 IU; see Table 7.5) administered at the peak of vulval swelling (diameter ≥ 15 mm) and repeated in 7–10 days if vulval regression does not occur.

Dosing

Gastric intubation is accomplished in fully conscious ferrets with a 10–16-gauge × 6 in. straight, stainless steel dosing needle with a 6.4 mm stainless steel ball on the end (EJAY International, Inc., Glendora, California) or, alternatively, with an appropriate diameter plastic tubing or French catheter. Because of the required length of the intubation needle or tubing, the animals should be held and treated in a vertical position. This is most easily accomplished by holding or resting the animal vertically across one's chest, allowing the intubation tube a straight, downward motion.

As with other test species, placement of the intubation tube within the stomach of the ferret is confirmed by visual examination of aspirate. McLain and Roe (1983) described the successful, daily intubation of approximately 40 pregnant female ferrets (20 doses/animal, 800 doses) without incident using this procedure.

Gag Reflex and Emesis in the Ferret

Similar to the dog and cat, the ferret is capable of a gag or vomit reflex and has received considerable attention as an alternative animal model for the study of the physiology of emesis (Florczyk et al., 1981). Oral dosing of nausea-producing agents will elicit characteristic behavior, including licking, chin rubbing, walking backward, and having slit eyes. Andrews et al. (1990) have published an excellent review of the abdominal visceral innervation and the emetic reflex in ferrets. Its similarity of responses to those of humans has made the ferret the model of choice for studying emesis (Sam et al., 2003; Schnell, 2003).

Gut Physiology and Microflora

Poddar and colleagues (Poddar, 1977; Poddar and Jacob, 1977; Poddar and Murgatroyd, 1976) and Pfeiffer (1970a,b) have published rather extensively on the morphological and histological similarities of the GI tract of the human, ferret, and monkey.

Past efforts have failed to identify any anaerobic gut microflora in the ferret (Gad, personal communication, August 1989). Identified enteric organisms include *Salmonella*, *Campylobacter* sp., *Cryptosporidium* sp., and *Eimeria* sp. Moody et al. (1985) concluded that the IV administration of a test article to ferrets is virtually impossible without an indwelling catheter because the species lack easily accessible veins. More recently, Fox (1988) reiterated this and suggested that venous access (jugular, cephalic, or femoral) in the ferret might require sedation, local anesthesia, and a surgical skin incision for placement of a catheter for short-term drug or fluid administration.

However, McLain et al. (1987) have reported on the subchronic (90 days, three times weekly), IV administration protocol in ferrets without the use of an indwelling catheter. Moreover, these authors routinely administer IV fluids repeatedly, and large volumes have been administered daily with prolonged infusion times by using either a 23-gauge, 1 in. needle, Angiocath or Quick-Cath catheter. These procedures are possible with either mechanical or physical restraint of test animals and by access of the caudal vein.

The caudal veins of the ferret cannot be palpated, nor are they visible to the naked eye. To access these veins, view the dorsal surface of the shaved tail as the 12 o'clock position. Turn the tail in a clockwise or counterclockwise direction so that either of the large veins residing at the 10 o'clock or 2 o'clock position now assumes that 12 o'clock position. Insert a 23-gauge, 1 in. needle (or, e.g., Quick-Cath catheter) at a depth of angle similar to that used for rodents. The caudal artery assumes at 6 o'clock position when the animal is viewed dorsally. A diagram of a transverse section of the ferret tail, showing location of caudal artery and veins, was published by Bleakley (1980).

Typical IV infusion volumes and rates employed for ferrets depend, as with other species, on the glomerular filtration rate (GFR) and the physical characteristics of the specific test article. Ferrets can probably receive between 2 and 4 mL/kg/hour of IV fluids without a notable increase in urine volume or frequency. This compares with an approximate continuous infusion rate of 0.5 mL/kg/hour for dogs and 8 mL/kg/hour for rats. Using the aforementioned ratios and "reference" body weights of 0.25, 1.5, and 10.0 kg for rat, ferret, and dog, respectively, the approximate total daily (24 hours) fluid volume that can be delivered to these test species without notable increases in the urine volume or frequency is, therefore, 50 mL (rat), 70–140 mL (ferret), and 120 mL (dog).

Humans (70 kg) typically can receive volumes up to 3000 mL/day, or approximately 2 mL/kg/hour. Increased fluid administration rates and volumes will generally be accommodated by all test species in direct relation to their individual GFRs.

Restraining Tube for Intravenous Dosing

Limited or initial IV dosing success might be possible with the crude ferret-restraining device originally proposed by Curl and Curl (1985) for serial blood sampling. However, as the IV access capabilities of the laboratory improve, and more prolonged and frequent infusions are required in animals of different sizes, a more sophisticated restraint system will be necessary.

The typical ferret-restraining device was designed for the purpose of accommodating the increasing skills of the laboratory's technical staff. Four different-sized restrainers have been developed and are proposed to accommodate the sexual dimorphism of ferrets, the seasonal deposition of body fat, and the various ages used in different research investigations. The inside diameters of the restraining tubes and the corresponding weight ranges that they have accommodated include 6.4 cm, 400–700 g; 7.9 cm, 650 to −1100 g; 8.8 cm, 1000 to −1750 g; and 10.1 cm, 1500–2300 g. Details of construction have been reported by McLain and McGrain-Dutson (1989).

Surgical Implantation and Maintenance of Indwelling Intravenous Catheters in Ferrets

Indwelling IV catheters can be implanted in ferrets when it is necessary to administer solutions at a very slow or constant rate using an infusion pump or for chronic studies requiring many consecutive days of treatment.

Greener and Gillies (1985) have developed a method to ensure catheter patency throughout the course of acute or chronic studies without the use of heparin. These authors fill the catheter lumen with saline solution and clamp the catheter with a rubber-shod hemostat at the end of each infusion. They then remove the syringe containing a blunt needle from the lumen of the tubing, insert an appropriately sized stainless steel blocking pin, and then remove the rubber-shod hemostat. When the stainless steel pin is pushed slightly further into the catheter, the saline fluid is forced to fill the lumen tip at the venous end, discouraging clot formation. A detailed description of catheter implantation and maintenance in ferrets was published by Greener and Gillies (1985). An earlier method for the chronic jugular catheterization of the ferret was published by Florczyk and Schurig (1981). Animals can be treated in restraining tubes or handheld and treated.

Inhalation Exposure of Ferrets

The pulmonary mechanics and physiology of the ferret lung as well as the potential of the species as an animal model for inhalation toxicology have been described quite thoroughly by Vinegar et al. (1979, 1982, 1985) and by Boyd and Mangos (1981). Noteworthy advantages of the use of ferrets in inhalation toxicology include the fact that because the ferret has more submucosal glands in the bronchial wall and an additional generation of terminal bronchioles, it is closer to the human lung than is the dog lung. Moreover, the disproportionately large lungs of the species can provide a useful tool for the studies of uptake, clearance, and deposition of aerosols, pulmonary blood flow, and diffusion. As suggested by Vinegar et al. (1985), they might also be good candidates for an isolated perfused lung preparation for metabolic studies and for regional lavage to look for markers of lung injury.

Physiological Measurements of the Ferret Lung

Physiological measurements were made by Vinegar et al. (1985) on anesthetized, tracheotomized, supine male ferrets. Six animals weighing 576 ± 12 g had tidal volumes (V_t) of 6.06 ± 0.30 mL, respiratory frequencies (f) of 26.7 ± 3.9/min, minute volume of 157.0 ± 14.8 mL/min, dynamic lung compliance (C-dyn) of 2.48 ± 0.21 mL/cmH$_2$O, and pulmonary resistance (R) of 22.56 ± 1.61 mL/cmH$_2$O/L/s. Measurements on nine ferrets (including the six just mentioned) revealed a total lung capacity (TLC) of 89 ± 5 mL, vital capacity (VC) of 87 ± 5 mL, expiratory reserve volume of 16 ± 2, and a functional residual capacity (FRC) of 17.8 ± 2.0 mL. Maximum expiratory flow–volume curves showed peak flows of 10.1 VC/s at 75% VC and flows of 8.4 and 5.4 VC/s at 50% and 25% VC.

The TLC of ferrets was estimated to be approximately 297% of what would be predicted for an animal of its size.

Miscellaneous Dosing Routes

Intramuscular, subcutaneous, intradermal, dermal, or intraperitoneal dosing procedures in the ferret are essentially no different than what would be practiced for other test species. Intramuscular administration, for example, is most easily given in the lateral aspect of the upper leg using a 28-gauge, ⅝ in. needle. Subcutaneous or intradermal administration can be given just above the shoulders on the dorsal aspect of the neck, taking care not to deposit lipophilic test materials in subcutaneous fat pads, as they will be poorly absorbed. A dermal application site (for an uncovered test material) that is inaccessible to the ferret is the dorsal aspect of the neck, immediately above the shoulders.

Intraperitoneal administration to ferrets is generally easier when performed by two people. One person should restrain the animal by securing the neck, shoulders, and front limbs with one hand while using the other hand to secure the hind limbs and position the animal on its back or against the holder's chest. The injection can be administered with an appropriate gauge needle through an elevated, pinched portion of skin located around midabdomen. Because ferret skin is very thick, care should be taken to assure that the test material is not deposited subcutaneously or intradermally.

Less frequently used routes of administration in the ferret include intracerebral, intraduodenal, intrapleural, intranasal, intratracheal, intrathoracic, intravaginal, and rectal. Apparently, there are no unique anatomical features of the ferret that would preclude the use of any of these routes when the appropriate procedures are employed. In that respect, Chimes (1994) describes a method for surgical implantation of a catheter for repeated administration of liquid into the lungs of ferrets. By varying the size of the tubing, the method could be adapted for rodents or other animals. Furthermore, some of the techniques used to accommodate animal growth could be usefully adapted for other chronic catheterization or cannulation methods.

Important Physical Parameters

Developmental Milestones

At birth, ferret kits weigh between 7 and 10 g (Shump and Shump, 1978; Willis and Barrow, 1971). The deciduous teeth begin to erupt at approximately 2 weeks of age and emerge completely through the gums at 18 days of age (Fox, 1988). Young ferrets will begin to consume moistened solid food as early as 2–3 weeks of age. They will begin to hear sound at approximately 32 days of age, and the eyes begin to open at approximately 34 days. The permanent canine teeth begin to appear at 47–52 days of age, and the deciduous canines are shed at approximately 56–70 days. Young ferrets are weaned at approximately 6–8 weeks postnatally.

Growth Curves and Typical Body Weight Ranges

At approximately 6–8 weeks of age a marked sexual dimorphism becomes apparent (Figure 7.2), with adult male body weights (reached at approximately 4–5 months) eventually exceeding approximately twice that of adult females. Under optimum controlled environmental conditions, sexually inactive adult male ferrets, or hobs, will generally weigh between 1 and 2 kg at 5–7 months of age. In contrast, anestrous females (jills) will generally weigh between 0.5 and 1.0 kg at this same age. The observed range in body weight of sexually inactive animals maintained under controlled conditions is largely dependent on the specific husbandry practiced, although the strain and source of supply (specific vendor) can also contribute.

Marked, seasonal body weight fluctuations as high as 30%–40% are commonly observed in both sexes and coincide with the natural breeding season of the species (March–August). Hobs and jills as young as 4–5 months of age will generally begin to deposit subcutaneous body fat in the fall (under natural lighting conditions) in preparation for the spring breeding season. Teleologically speaking, this conformational change probably evolved to facilitate propagation and survival of the species. Under natural conditions, for example, sexually active male ferrets might ignore food for extended periods in their concentrated search for receptive females. In addition, the prolonged coitus (several hours) of the species and the frequently fatal exchange following the chance encounter of two sexually active, territorial males can require tremendous amounts of energy. Males will generally return to, or remain at, a sexually inactive (undescended testicles) body weight under controlled lighting conditions. Females, on the other hand, because they are induced ovulators, can manifest a prolonged estrus with diminished appetite and subsequent marked body weight loss. Optimum environmental lighting (see earlier) and hormonal injections (see earlier) will preclude the onset of bone marrow hypoplasia or pometra associated with prolonged estrus in this species (Sherrill and Gorham, 1985).
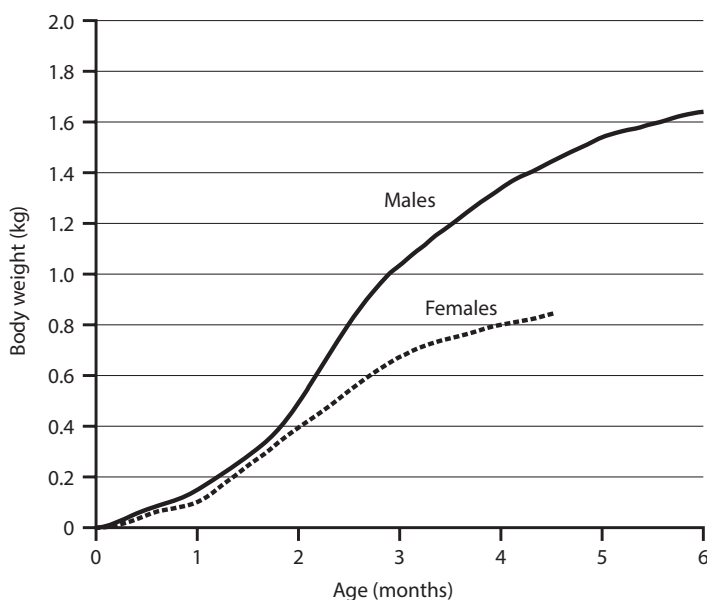
Figure 7.2   Growth curve for ferrets. A newborn kit's body weight will range between 6 and 12 g at birth. At approximately 8 weeks, the sexual dimorphism of the species begins, with male ferrets eventually attaining an adult body weight approximately twice that of females.

Age at Maturation

Male and female ferrets are generally assumed to be sexually mature 6–8 months of age. However, this will depend to a great extent on the photoperiodicity to which the individual animals are exposed (natural vs. controlled) or to the month (season) in which they are born. It is possible, for example, for animals as young as 4 months of age to conceive and produce viable offspring.

Although rats are sexually (i.e., physiologically) mature at 50–60 days of age (Rowett, 1965), they are not routinely first mated until approximately 100–120 days of age so that reproductive performance is maximized. McLain et al. (1985b) have demonstrated that reproductive performance in primiparous ferrets is maximized at approximately 7–10 months of age and decreases thereafter. Consequently, although physiological sexual maturity might occur earlier, 7–10 months is probably an optimum breeding age for ferrets.

Weight and Appearance of Testes

The male ferret's potential breeding season under natural lighting conditions extends from December to July, which precedes the female's breeding season of approximately March through August. Ryland and Gorham (1978) attribute this earlier sexual maturation of the male to a functional adaptation to allow for adequate sperm maturation.

Ishida (1968) and Basrur and Gilman (1968) also described the age and seasonal changes in the testes of the ferret. In ferrets born in June (natural lighting), bilateral testicular development started in December and reached maturity by February, with the functional period lasting from March until July and the period of quiescence being from August until December.

Infantile ferret testes are characterized by the lack of germinal cycle, by undifferentiated precursors of Sertoli cells, by the absence of a tubular lumen, and by small interstitial cells. The prepubertal testes become large as a result of the development of the germinal epithelium and tubular lumen and by virtue of the growth of the Sertoli and interstitial cells (Boissin-Agasse and Boissin, 1979; Ishida, 1968). The testes can range in weight from 0.8 to 3.8 g/kg, with a mean weight of approximately 2.02 g/kg (Fox, 1988).

The male ferret lacks seminal vesicles and vulvourethral glands, Moreover, the presence of a prostate gland in the ferret has been a subject of debate, and, as in the majority of male mustelids, it has been classified as poorly developed (see Mead [1970] for reviews). However, during the first several months of postnatal development of the male ferret and during its sexual quiescence, prostatic tissue is difficult to locate except through histological examination. The prostate of the adult, sexually active ferret, on the other hand, is visible at gross autopsy.

The ventrally located penis is large for the animal's size, with the distal section curving dorsally so that it ends in a hook (Moody et al., 1985). The testes are located in the subcutaneous tissue of the caudoventral abdomen and descend into the scrotum only during the breeding season.

Curry et al. (1989) have published a comparison of sperm morphology and silver nitrate staining characteristics in the domestic ferret and the black-footed ferret. Shump et al. (1976) have previously described the semen volume and sperm concentration in the ferret.

Frequency of Estrus

Female ferrets are seasonally polyestrous and will generally reach sexual maturation at approximately the same age as males. Estrus is induced by increased day length or by manipulation of the photoperiod with artificial illumination (described earlier). The onset of estrus in female ferrets is recognized by a continuous vulval swelling occurring over an approximate 2–3-week period. The vulva will increase in size approximately 10-fold, measuring approximately 1.0–1.5 cm in

diameter in primiparous females and approximately 1.5–2.0 cm in diameter in multiparous animals at peak receptivity. Mating should occur at the peak of vulval swelling (approximately 2 weeks after the onset), when the vulva has a slightly pink and mucous-covered appearance. Ovulation is induced in the ferret approximately 3–36 hours after coitus, with implantation occurring approximately 12–13 days postcoitus. The vulva will begin to regress approximately 3–4 days after mating, regaining its proestrus size in approximately 2–3 weeks.

As indicated previously, female ferrets are induced ovulators and will remain in estrus for as long as 6 months if not mated. Prolonged estrus in this species can precipitate pyometra or complications of bone marrow hypoplasia and estrogen-induced anemia (described earlier). Females will generally return to estrus approximately 2 weeks after weaning of the litter (McLain et al., 1985b). Occasionally, a lactation estrus will occur in females with less than five suckling young (Fox, 1988).

Gestational Period and Litter Size

McLain et al. (1985b) analyzed demographic data for 945 female ferrets from a commercial breeding colony for the effects of various maternal characteristics on subsequent reproductive performance. In general, litter size ($M \pm SEM$) was found to be greatest for young, primiparous females (10.3 ± 0.2) and decreased with advanced maternal age and parity to a cohort mean of 8.1 ± 0.1 for third-parity females 16 months of age. Gestational length ($M \pm SD$) was 41.3 ± 1.1 days and appeared to decrease with increasing day length and total litter size. The gestation period in this cohort ranged from 39 (observed in 32 primiparous jills) to 46 days (observed in 4 multiparous females). Reproductive data for ferrets are summarized in Table 7.6.

Table 7.6   Typical Parameters of Reproduction in Ferrets Maintained under Controlled Environmental Conditions

Parameter	Units	Value
Age at pairing, M/F	Months	8–10
Breeding life, M/F	Years	2–5
Breeding season, M/F	By photoperiod	All year
Type of estrus cycle	—	Monoestrus
Duration of estrus	Days	Prolonged
Copulation time	Hours	Up to 3
Sperm deposition site	—	Posterior cervix
Sperm capacitation	Hours	3–11
Sperm viability	Hours in tract	36–48
Mechanism of ovulation	—	Induced
Time of ovulation	Hours, postcoitus	30–36
No. of ova	Average	12, range = 5–18
Ovum transit time	Days, postcoitus	5–6
Time of implantation	Days, postcoitus	12–13
Length of gestation	Days	41, range = 39–46
Litter size	Average	8–10, range = 1–18
Weight at birth	g	8, range = 6–12
Age at weaning	Weeks	6
Weight at weaning	kg	0.2–0.4
Rebreeding	—	Immediately

Source: Data from Fox, J. G., *Biology and Diseases of the Ferret*, Philadelphia, PA: Lea & Febiger, 1988; Marshall, K. R. and Marshall, G. W. *The Biomedical Use of Ferrets in Research (Supplement 1)*, North Rose, NY: Marshall Research Animals, Inc., 1973; McLain, D. E. et al., *Lab. Anim. Sci.*, 35, 251, 1985b; Moody, K. D. et al., *Lab. Anim. Sci.*, 35, 272, 1985.

Normative Physiological Data

Studies of the effects and mechanisms of experimental treatments require that substantial normative physiological data for the species be established. Thus, as a contemporary animal model, the ferret continues to benefit from ongoing investigations contributing to this monumental task. Normative physiological data also aid in test species selection because in addition to its particular similarities with man, toxicologists will frequently select an animal model because the system of interest can be externally manipulated or adapted to operate under extreme conditions. In this respect, much of the normative physiological data generated for ferrets have proven rewarding in that several potentially superior systems have been identified. On the other hand, the same contemporaries of ferrets continue to leave many questions unanswered.

Table 7.7 provides available normal cardiopulmonary data for the ferret.

Cardiovascular Parameters

Earlier discussions of the ferret's cardiovascular system were offered by Kempf and Chang (1949, macroscopic) and Borelli and Filho (1971, microscopic). Later reviews include those by Andrews et al. (1979b), Baskin et al. (1981), and Andrews (1988).

Heart rate and blood pressure measurements have been made in both anesthetized and unanesthetized ferrets, but considerable more data have been generated for anesthetized animals. An apparent anesthetic effect on the heart rate is evident by the differences reported by Andrews et al. (1979b)

Table 7.7 Selected Normative Cardiopulmonary Data for Ferrets Maintained under Controlled Environmental Conditions

Parameter	Units	Value
Cardiovascular		
Blood volume	mL/kg	60–70
Blood pressure		
Systolic	mmHg	140–164
Diastolic	mmHg	110–125
Cardiac output	mL/min	139 (range = 82–200)
Circulation time		
Fluorescein	s	6.8 ± 1.2
Cyanide	s	4.5 ± 0.7
Heart rate		
Barbiturate	bpm	230 ± 26
Halothane	bpm	387 ± 54
Conscious, active	bpm	341 ± 39
Conscious, inactive	bpm	200–255
Pulmonary		
Tidal volume	mL	6.06 ± 0.30
Respiration rate		
Conscious	bpm	33–36
Pentobarbital	bpm	26.7 ± 3.9
Dynamic compliance	mL/cmH$_2$O	2.48 ± 0.21
Pulmonary resistance	cmH$_2$O/L/s	22.56 ± 1.61
Total capacity	mL	89 ± 5
FRC	mL	17.8 ± 2.0

Source: Data from Andrews, P. L. R. et al., *Lab. Anim.*, 13, 215, 1979b; Thornton, P. C. et al., *Lab. Anim.*, 13, 119, 1979; Kempf, J. E. and Chang, H. T., *Proc. Soc. Exp. Biol. Med.*, 72, 711, 1949; Andrews, P. L. R., The physiology of the ferret. In *Biology and Diseases of the Ferret*, ed. J. G. Fox, Lea and Febiger, Philadelphia, PA, 1988; Vinegar, A. et al., *Lab. Anim. Sci.*, 35, 246, 1985.

for animals under urethane anesthesia (387 ± 54 beats/min [bpm]) and that reported by Thornton et al. (1979) for animals under barbiturate anesthesia (230 ± 26 bpm). In the unanesthetized animal, heart rates have been reported to range from 341 ± 39 (Andrews et al., 1979b) to 200–255 bpm (Thornton et al., 1979). Smith and Bishop (1985) have reported similar disparities in the heart rate (bpm) of anesthetized (sodium pentobarbital) adult control ferrets (309, range = 250–380), anesthetized (sodium pentobarbital) adult ferrets with right ventricular hypertrophy (300, range = 245–380) and anesthetized (KET) weanling ferrets (280, range = 210–360). Andrews and Illman (1987) have suggested that the problem of variations in the heart rate of the ferret could be resolved by recording the animal's activity level and by viewing the results under different anesthetics as two ends of a spectrum, between which the heart rate of the unanesthetized animal would operate according to the animal's activity level.

Apparently, Kempf and Chang (1949) have provided the only measurement of cardiac output measured 139 mL/min (range = 82–200 mL/min) and circulation time measured 6.8 ± 1.2 s (fluorescein) and 4.5 ± 0.7 s (cyanide). Under urethane or barbiturate anesthesia, mean systolic blood pressure values between 140 and 164 mmHg have been reported from the ferret, with diastolic values of 110–125 mmHg (Andrews et al., 1979b; Kempf and Chang, 1949). In the conscious animal, systolic values have been reported as 161 mmHg (males) and 133 mmHg (females), with some animals presenting with blood pressure values as high as 190 mmHg (Thornton et al., 1979).

The electrocardiogram of normal ferrets and ferrets with right ventricular hypertrophy has been reported by Smith and Bishop (1985). These authors concluded that the normal ferret has a mean electrical axis of +86° ± 6.6 (SD), with a narrow range between +69° and +97°. All animals in their study exhibited a normal sinus rhythm, composed of the expected P wave, QRS complex, and T wave.

Experience from our laboratory has indicated that a maximum of approximately 50%–60% of a ferret's total blood volume be removed by cardiac puncture technique (exsanguination). Therefore, estimates suggest that the ferret's total blood volume is 6%–7% of the body weight, or approximately 60–70 mL/kg.

Pulmonary Parameters

An early study by Barer et al. (1978) and a species comparison study by Peake et al. (1981) investigated the response of the blood-perfused ferret lung to hypoxia. Both groups of authors concluded that of several species investigated, the ferret has the most marked pulmonary vasoconstriction in response to hypoxia, with a maximum response occurring at approximately 25 mmHg. Vinegar et al. (1982) have postulated that the sensitivity to hypoxia in the ferret might be an adaptation to burrowing. The sensitivity of the ferret's pulmonary vasculature to hypoxia makes it a valuable model for the study of pulmonary hypertension in humans. A descriptive pulmonary vascular pressure profile in adult ferrets (in vivo and in isolated lungs) has been reported by Raj et al. (1990a), and in 2–3-week-old, 5–6-week-old, and adult ferrets (Raj et al., 1990b).

In addition to the extensive data generated by Vinegar et al. (1985), discussed previously, various other investigators have examined the respiratory rate of ferrets. For example, Pyle (1940) reported the rate to be 33–36 breaths/min in the conscious ferret as compared to values of 31 ± 6 breaths/min in urethane-anesthetized animals (Andrews et al., 1979b) and 43.5 ± 4.6 breaths/min in pentobarbital-anesthetized animals (Boyd and Mangos, 1981). Similar to the results in the cardiovascular system, the disparity in reported respiration rates of the ferret might be the result of differences in type or dosage of anesthesia, or the age of the animals. Scientific protocols should, therefore, include requirements for documentation of these variables.

Normative pulmonary and cardiovascular values for ferrets are summarized in Table 7.7.

Clinical Laboratory Parameters

Data for hematological serum chemistry determinations in ferrets have been reported by Thornton et al. (1979), Lee et al. (1982), Moody et al. (1985), and Fox (1988). However, all of these studies used small numbers of animals, and only Lee and colleagues described the variability in their sample of five females and eight males (three intact, five castrated). Fox (1988) contrasted the analytical results obtained in his laboratory from both orbital plexus and cardiac blood sample sites in ferrets. However, the data were not expressed statistically, and an estimation of variability was not provided. To date, therefore, no studies have satisfactorily described the hemogram of laboratory ferrets with respect to sample variability and the distribution around the mean. Moreover, because Neptun et al. (1985, 1986) concluded that both sampling site and collection method are major sources of variation in clinical laboratory measurements of homogeneous laboratory rats, the potential for sample site and collection method differences must be adequately evaluated in ferrets. Obviously, when differences can be demonstrated, selection of an appropriate collection method and sampling site should include a consideration of which parameters are likely to be of major interest. For heterogeneous species such as the ferret, this would seem especially important.

In our experience, a significant amount of clinical laboratory data have been personally accumulated from ferrets used in acute and subacute GLP testing protocols. Moreover, the experimental results generated with these animals have been submitted to the FDA in fulfillment of the requirement for test data generated in a second species. In all probability, the sound statistical treatment of the data generated in these studies (McLain and Lin, 1989), which is deemed necessary and appropriate for normalization of any heterogeneous species, was well received by the FDA reviewers and contributed significantly to the success of each submission process. Therefore, these data and discussion of their statistical treatment are summarized in the following sections.

Blood Sample Collection

Blood samples for clinical laboratory determinations in ferrets can be collected by several routes. Cardiac puncture and orbital plexus sampling of anesthetized animals might be the most rapid and efficient method when large numbers of samples are to be processed. However, alternative sites include toenail clipping, jugular venipuncture, and tail vein and artery for repeated sampling such as in pharmacokinetic analyses (McLain et al., 1985a). Currently, the collection by the saphenous vein is the preferred methodology for repeat sampling during a study (Hem et al., 1998).

Hematological Parameters

In construction of the following database, blood for clinical pathology was collected in tubes containing either sodium citrate (3.8%) or potassium ethylenediaminetetraacetate (EDTA). Blood collected in tubes containing sodium citrate was used for the determination of prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen level. Blood collected in tubes containing EDTA was used for determination of all other hematological parameters.

Hematological determinations collected before experimental treatment in 370 adult (6–10 months) ferrets (187 males, 183 females) are summarized in Tables 7.8 and 7.9. Reference values for the clotting factors (APTT, PT) were determined after IV saline treatment (single dose) of 64 animals (32 males, 32 females) from the preceding cohort and from 32 additional animals (16 males, 16 females) that served as untreated (UT) controls in the aforementioned cohort. All ferrets were obtained from the same vendor. Fasted blood samples were collected from anesthetized animals (KET–ACE, 10:1, 35 mg/kg intramuscular) via cardiac puncture and assayed by conventional methods. Approximately 1.3% of the samples collected for hematology were not

Table 7.8   Hematology Values for Adult Ferrets Maintained under Controlled Environmental Conditions

Parameter	Sex	*M*	*SD*	N	Min	Max	CT	TL Lower	TL Upper	Method	Sex Effect	Transformation
Hgb (g/dL)	F	16.2	1.3	180	10.9	19.0	16.2	13.5	18.9	P	$p < .001$	None
	M	16.8	1.2	185	13.3	20.0	16.8	14.3	19.3			
HCT (%)	F	48.4	4.0	180	33.2	57.8	48.4	40.0	56.8	P	$p < .01$	None
	M	49.8	3.7	185	39.6	62.0	49.8	42.0	57.5			
RBCs (mil/mm³)	F	9.30	0.84	180	5.77	11.52	9.30	7.52	11.08	P	$p < .001$	None
	M	9.69	0.71	185	7.95	11.86	9.69	8.18	11.19			
MCV (µ³)	F	52.2	2.0	180	42.5	60.3	52.2	48.0	56.4	P	$p < .001$	None
	M	51.4	1.4	185	47.9	54.9	51.4	48.4	54.4			
MCH (pg)	F	17.5	0.7	180	14.8	20.5	17.5	16.0	19.0	P	ns	None
	M	17.3	0.7	185	15.6	19.2	17.3	15.9	18.7			
MCHC (%)	F	33.5	0.9	180	30.5	36.7	33.5	31.6	35.4	P	$p < .05$	None
	M	33.8	0.9	185	31.5	36.1	33.8	31.8	35.8			
Platelet (10³/mm³)	F	764	246	157	330	1520	726	364	1447	P	ns	Ln(X)
	M	766	196	163	315	1525	742	438	1256			
Fibrinogen (mg/dL)	F	184	66	182	83	548	170	99	376	P	ns	Ln(X − 63)
	M	189	28	187	93	657	173	100	387			
WBCs (10³/mm³)	F	7.6	3.2	180	2.9	23.1	7.0	3.0	16.3	P	$p < .001$	Ln(X)
	M	9.2	2.7	185	3.7	18.2	8.8	4.6	16.6			
Lymphocytes (10³/mm³)	F	3.4	1.8	180	1.0	11.1	3.0	1.1	8.2	P	$p < .001$	Ln(X)
	M	4.2	1.6	185	1.2	8.3	3.9	1.7	9.0			
Polymorphic neutrophils (PMNs) (10³/mm³)	F	3.8	2.0	180	1.2	13.3	3.4	1.2	9.4	P	$p < .001$	Ln(X)
	M	4.5	2.0	185	1.4	13.0	4.1	1.7	9.9			
EOS (10³/mm³)	F	0.25	0.22	180	0.00	1.39	0.18	0.00	0.78	N	$p < .01$	NA
	M	0.33	0.28	185	0.00	2.13	0.25	0.00	0.92			
MONO (10³/mm³)	F	0.09	0.13	180	0.00	0.86	0.05	0.00	0.48	N	ns	NA
	M	0.11	0.13	185	0.00	0.84	0.09	0.00	0.37			
BASO (10³/mm³)	F	0.03	0.07	180	0.00	0.46	0.00	0.00	0.23	N	$p < .05$	NA
	M	0.04	0.08	185	0.00	0.49	0.00	0.00	0.25			
STAB (10³/mm³)	F	0.01	0.03	180	0.00	0.22	0.00	0.00	0.13	N	ns	NA
	M	0.01	0.05	185	0.00	0.40	0.00	0.00	0.14			

Note: P, parametric; N, nonparametric; ns, not significant; NA, not applicable; Ln, natural logarithm; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; EOS, eosinophils; MONO, monocytes; BASO, basophils; STAB, band neutrophils.

analyzed for various reasons. Approximately 13.5% of the platelet samples were "clumped" and not suitable for analysis. Only 1 of 370 fibrinogen samples collected was not reported. Mortality associated with this blood collection procedure was generally low (approximately 2%), occurred largely in females, and was apparently correlated with the quantity of blood withdrawn from the animal (10 mL/animal, or approximately 10% and 20% of the total blood volume of males and females, respectively).

Methodological parameters used on the Coulter hemoglobinometer, Coulter ZBI, MLA 700, or by microscope for determination of hematology reference values included APTT (clotting time, cephaloplastin), PT (clotting time, thromboplastin), hemoglobin (Hgb, Coulter Hemoterge II),

Table 7.9 Whole Blood Clotting Factors, Serum Electrolytes, and Serum Enzyme Levels for Adult Ferrets Maintained under Controlled Environmental Conditions

Parameter	Sex	*M*	*SD*	N	Min	Max	CT	TL Lower	Upper	Method	Sex Effect	Transformation
Blood clotting factors												
PT (s)	M + F	11.1	0.4	94	10.3	12.4	11.0	10.3	12.1	P	ns	Ln(X − 9)
APTT (s)	M + F	20.9	24.5	94	13.9	200.0	17.1	14.8	21.2	P	ns	Ln(X − 11.8)
Serum electrolytes												
Na (mEq/L)	M + F	150	96	96	135	159	150	141	159	P	ns	None
K (mEq/L)	M + F	4.76	96	96	3.90	6.80	4.70	4.09	5.97	P	ns	Ln(X − 3.5)
Cl (mEq/L)	M + F	114	96	96	96	124	114	108	120	P	ns	None
Serum enzymes												
Alkaline phosphatase (IU/L)	F	40	18	183	13	106	35	18	92	P	ns	Ln(X − 10)
	M	37	18	187	15	99	33	17	88			
LDH (IU/L)	F	621	407	183	174	2030	476	177	2,341	P	ns	Ln(X − 120)
	M	595	505	187	143	3030	426	163	2,309			
SGOT (IU/L)	F	97	48	183	34	271	82	41	282	P	ns	Ln(X − 30)
	M	92	56	187	36	442	78	41	236			
SGPT (IU/L)	F	210	226	183	47	1710	146	55	765	P	ns	Ln(X − 40)
	M	205	207	187	45	1850	153	60	670			

Note: P, parametric; ns, not significant; Ln, natural logarithm.

hematocrit (HCT, calculated), erythrocytes (red blood cells [RBCs], electrical resistance), mean corpuscular volume (MCV, electrical resistance), mean corpuscular hemoglobin (MCH, calculated), mean corpuscular hemoglobin concentration (MCHC, calculated), platelet count (Unopette, manual), fibrinogen (optics), leukocytes (white blood cells [WBC], electrical resistance), and lymphocyte count, PMN count, eosinophils (EOS), monocytes (MONO), basophils (BASO), and band neutrophils (STAB) by Wright–Giemsa stain.

Noteworthy observation in sample collection included the fact that many citrate tubes were hemolyzed and a large percentage of platelet samples were clumped. Additionally, a manual method, which takes three times longer than the automated method, was necessary for analysis of platelets because of their large size. Finally, relative to other species, ferrets have many RBCs, and blood samples will require a dilution before running on the Coulter ZBI. An additional 20%–80% might require further dilution.

Table 7.8 lists the calculated reference values for selected hematology measurements of the ferret. The first column lists the various parameters and their units. The third through seventh columns list the descriptive statistics, which include the mean, standard deviation (*SD*), number of observations (*N*), minimum value (Min), and maximum value (Max). These descriptive statistics were based on the original units (scale) and were not affected by the transformation, if needed. The last column lists the transformation, if needed, for skewed distributions of measurement to become approximately normally (Gaussian) distributed. The eighth column lists the central tendency (CT) of each parameter. The CT is the antitransformation of the transformed mean. For example, it is the geometrical mean if a logarithmic transformation is applied. If a transformation is needed, the CT better represents the norm of the population than

the mean would. The next two columns list the TLs. The TLs contain 95% of the measurements (in the original scale) of UT ferrets with 90% confidence. The next to the last column lists the test on the difference between means of males and females. The column to its left explains whether the TLs and the sex effect were determined by parametric (P) or nonparametric (N) methods.

Reference values for the clotting factors (PT, APTT) are listed separately with the electrolytes and serum proteins (Table 7.9). Unlike the values in Table 7.8, these reference values are listed combining males and females. This was done because the database was much smaller, and no sex differences were detected for any of the parameters.

By comparison, the variance observed in ferret hematology is approximately one-half of that reported for humans (Conn, 1963), minimally greater than the beagle dog and considerably greater than the inbred SPF and virus-free rat (see specific chapters). Notable exceptions (increases or decreases) in mean values for hematological parameters in ferrets relative to humans include (males only, ferret/human) RBC (million/mm^3), 9.69 $\pm$ 0.71/5.4 $\pm$ 0.8 and MCV (μ^3), 438–1236/150–450. Thornton et al. (1979) observed a higher HCT (and RBCs) in ferrets relative to rats and dogs and recommended a 20% longer spin time (due to a negligible RBC sedimentation rate of the ferret) for blood samples used for microhematocrit determination. These authors also reported a higher Hgb level in males (55.4, $n = 28$) relative to females (49.2, $n = 11$) and no sex difference in number of WBCs (or differential). The present database confirmed a high HCT and Hgb in ferrets. However, the sex difference in Hgb, although still significant, is apparently not as large when cardiac blood samples are used or when the sample size is increased. Furthermore, the observations in this study that male ferrets have significantly greater numbers of WBCs (including lymphocytes, polymorphonuclear neutrophils, EOS, and BASO) than females are in contrast to what Thornton et al. (1979) have reported and in agreement with Lee et al. (1982).

Serum Chemistry Parameters

Reference serum chemistry values obtained from the same population of animals are listed in Table 7.10. The blood collection procedure and table format are the same as previously described. Serum electrolytes and enzymes are included in Table 7.9 with the clotting factors.

Methodological procedures used on the Hitachi 705 Chemistry Analyzer for determination of reference values include albumin (bromocresol green), alkaline phosphatase (modified Bowers/McComb), serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) (modified Henry), BUN (urease), calcium (CPC), chloride (Beckman E4), cholesterol (esterase/oxidase), creatinine (Jaffe), glucose (hexokinase), lactic dehydrogenase (LDH) (modified Wacker), Na and K (Beckman E4), phosphorus (molybdate), total bilirubin (DPD), total globulin and A/G ratio (calculated), total protein (Biuret), and uric acid (uricase).

Similar to the hematological findings, the variance observed in the ferret clinical chemistry is minimally greater than the beagle dog and considerably greater than the inbred SPF and virus-free rat (see specific chapters). Notable exceptions (increases or decreases) in the range in values for serum chemistry parameters in ferrets relative to humans (Conn, 1963) include (males only, ferret/human) glucose (fasting, mg/dL), 81–142/60–100; BUN (mg/dL), 13–37/10–20; uric acid (mg/dL), 0.4–2.8/3.0–6.0; creatinine (mg/dL), 0.20–0.70/0.7–1.5; SGOT (IU/dL), 41–236/5–40; and SGPT (IU/dL), 17–88/5–13. The considerable range in clinical chemistry parameters of the ferret, especially the liver enzymes, obviates the need for an analysis of the "change from baseline" rather than any particular absolute value in this species. Moreover, appropriate transformations should be applied whenever possible to all posttreatment values adjusted for pretreatment measurements.

Table 7.10 Serum Chemistry Values for Adult Ferrets Maintained in a Controlled Environment

Parameter	Sex	*M*	*SD*	N	Min	Max	CT	TL Lower	TL Upper	Method	Sex Effect	Transformation
Calcium (mg/dL)	F	9.6	0.9	183	5.0	11.0	9.6	8.0	11.2	P	ns	None
	M	9.5	0.7	187	6.9	11.2	9.5	8.0	11.1			
Phosphorus (mg/dL)	F	6.5	1.0	183	4.0	8.7	6.5	4.4	8.5	P	$p < .001$	None
	M	7.0	1.0	187	4.9	9.5	7.0	5.0	9.0			
Glucose (mg/dL)	F	118	25	183	62	387	116	85	152	P	$p < .001$	$Ln(X + 96)$
	M	110	15	187	28	164	110	81	142			
BUN (mg/dL)	F	27	7	187	12	76	20	13	37	P	$p < .001$	$Ln(X - 9)$
	M	21	7	187	12	76	20	13	37			
Uric acid (mg/dL)	F	1.8	0.5	183	0.5	3.5	1.7	0.8	3.2	P	$p < .001$	$Ln(X + 0.74)$
	M	1.3	0.6	187	0.2	3.0	1.3	0.4	2.8			
Cholesterol (mg/dL)	F	183	33	183	96	269	183	112	254	P	$p < .001$	None
	M	162	25	187	107	238	162	108	215			
Bilirubin (mg/dL)	F	0.2	0.1	183	0.0	0.4	0.2	0.0	0.3	N	ns	NA
	M	0.2	0.1	187	0.0	0.4	0.2	0.0	0.3			
Creatinine (mg/dL)	F	0.44	0.12	183	0.30	1.00	0.40	0.20	0.70	N	$p < .001$	NA
	M	0.48	0.10	187	0.20	1.00	0.50	0.20	0.70			
Total protein (g/dL)	F	5.9	0.8	183	3.5	9.7	5.9	4.3	7.6	P	ns	None
	M	6.0	0.8	187	3.9	11.2	6.0	4.6	7.4			
Albumin (g/dL)	F	3.4	0.5	183	1.8	5.7	3.4	2.4	4.4	P	ns	None
	M	3.5	0.4	187	2.2	4.5	3.5	2.6	4.4			
Globulin (g/dL)	F	2.5	0.5	183	1.2	5.5	2.4	1.8	3.8	P	ns	$Ln(X - 1.23)$
	M	2.6	0.7	187	1.7	8.4	2.4	1.8	3.8			
A/G ratio	F	1.39	0.28	183	0.56	3.25	1.40	0.80	1.82	P	ns	X^2
	M	1.42	0.29	187	0.33	2.05	1.45	0.72	1.92			
Bilirubin (mg/dL)	F	0.2	0.1	183	0.0	0.4	0.2	0.0	0.3	N	ns	NA
	M	0.2	0.1	187	0.0	0.4	0.2	0.0	0.3			

Note: P, parametric; N, nonparametric; ns, not significant; NA, not applicable; Ln, natural logarithm; BUN, urease.

Effect of Sampling Site on Variations in Baseline Clinical Pathology Parameters of the Ferret

Tables 7.11 and 7.12 describe the influence of sample collection site on the variance of certain blood parameters of the ferret. In this analysis, the variances of select parameters of samples collected by the cardiac puncture technique described previously ($n = 370$) were compared to the variation in pretreatment blood samples, collected by orbital plexus technique, from 58 additional ferrets. The sex effect was removed in this comparison (i.e., the variance was pooled across sexes). The *p* value in Tables 7.11 and 7.12 is for comparing variances between collection sites.

For all of the hematology parameters listed in Table 7.11, except WBCs and BASO, the sample variance was significantly increased when collected by the orbital plexus relative to cardiac puncture. Moreover, when the critical level of significance is set at $\alpha = 0.05$, only EOS collected by each technique did not differ. Based on the number of clotted hematology samples in this analysis (12/58), however, as well as personal observations, it is recommended that each laboratory confirms its collection proficiency by this route in this species before protocol collection is initiated. In addition, the acceptability of each sample should be confirmed before an animal is returned to the home cage.

Table 7.11   Effect of Sampling Site on Variations in Baseline Hematology Parameters of Ferrets

Parameters[a]	No. of Samples[b]	Cardiac Puncture		Orbital Plexus		p Value
		M	SD	M	SD	
Hgb, g/dL	365/37	16.50	1.46	17.90	1.79	.00000
HCT, %	365/37	49.10	3.82	54.80	6.39	.00000
RBC, 10^6/mm^3	365/37	9.50	0.78	10.53	1.01	.00000
MCV, mm^3	365/37	51.80	1.73	52.00	3.02	.00000
MCH, pg	365/37	17.40	0.71	17.00	0.77	.02304
MCHC, %	365/37	33.70	0.95	32.80	1.10	.00088
Platelet, 10^3/mm^3	320/27	6.60	0.28	6.30	0.37	.00000
WBC, 10^3/mm^3	365/37	2.06	0.36	2.48	0.28	.04223
Lymphocytes, %	365/37	45.90	12.15	47.80	14.07	.00274
STAB, %	365/37	0.14	0.44	0.34	0.95	.00000
MONO, %	365/37	1.16	1.31	2.6	1.83	.00000
EOS, %	365/37	3.41	2.42	2.90	2.59	.08827
BASO, %	365/37	0.41	0.74	0.05	0.23	.00000
POLY, %	365/37	49.10	12.28	46.50	13.57	.02803

[a] Transformed values include platelet = Ln(X) and WBC = Ln(X).
[b] No. of samples = cardiac puncture plexus.

Table 7.12   Effect of Sampling Site on Variations in Baseline Serum Chemistry Parameters of Ferrets

Parameters[a]	No. of Samples[b]	Cardiac Puncture		Orbital Plexus		p Value
		M	SD	M	SD	
Calcium, mg/dL	368/57	9.59	0.74	9.90	0.55	.00329
Phosphorus, mg/dL	370/57	6.72	0.97	8.60	1.12	.00346
Glucose, mg/dL	369/57	5.35	0.10	5.33	0.17	.00000
Cholesterol, mg/dL	370/57	173	29.7	185	30.60	.28056
BUN, mg/dL	369/57	2.51	0.59	2.53	0.69	.00169
Uric acid, mg/dL	370/56	0.80	0.25	1.17	0.18	.00222
Bilirubin, mg/dL	370/58	0.17	0.10	0.06	0.13	.00001
Creatinine, mg/dL	370/57	0.46	0.11	0.51	0.13	.00012
Total protein, mg/dL	369/57	5.96	0.73	6.20	0.40	.00000
Albumin, mg/dL	370/57	3.44	0.46	3.69	0.30	.00014
Globulin, mg/dL	368/57	0.19	0.36	0.24	0.26	.00241
A/G ratio	368/57	2.04	0.69	2.33	0.64	.24671
Alkaline phosphatase, IU/l	370/58	3.18	0.57	3.32	0.30	.00000
LDH, IU/l	370/58	5.80	0.90	6.67	0.62	.00064
SGOT, IU/l	370/58	3.91	0.72	4.24	0.52	.00158
SGPT, IU/l	370/58	4.70	0.87	4.61	0.60	.00053

[a] Transformed values include glucose = Ln(X + 69); BUN = Ln(X – 9); uric acid = Ln(X + 0.74); globulin = Ln(X – 1.23);
    A/G Ratio = X2, alkaline phosphatase Ln(X – 10); LDH = Ln(X – 120); SGOT = Ln(X – 30); and SGPT = Ln(X – 40).
[b] No. of samples = cardiac puncture/orbital plexus.

For the 16 clinical chemistry parameters in Table 7.12, 8 parameters (uric acid, calcium, total protein, albumin, globulin, alkaline phosphatase, LDH, and SGPT), especially the liver enzymes, showed significantly smaller variances when collected from the orbital plexus. Conversely, five parameters (BUN, creatinine, bilirubin, glucose, and phosphorus) showed significantly larger variance, and cholesterol, SGOT, and A/G showed no significant difference.

Although reference to previously published mean values for this species is inappropriate, the orbital plexus appears to provide hematological and clinical chemistry mean values similar to those

reported by Thornton et al. (1979) for samples collected from the abdominal aorta. A much larger database would, however, have to be examined to confirm this.

Data from the foregoing analysis indicate that if protocols specify that blood samples are to be collected from multiple sites (i.e., cardiac puncture for hematology and orbital plexus for clinical chemistry), reductions in sample variance can be expected in most parameters, especially the liver enzymes. Alternatively, one can choose to optimize the ability to detect subtle changes in a select parameter by collecting a blood sample from the site affording the least variability.

Estimation of Ferret Sample Size Requirements for Optimum Statistical Power

McLain and Lin (1989) demonstrated that, when it is desired to achieve a statistical power in ferrets similar to that enjoyed with the less heterogeneous beagle dog, the needed sample size to detect a prespecified difference with prespecified statistical power and Type I error is proportional to the pure variance (random error). Therefore, the ferret's sample size requirements for any select parameter relative to the beagle dog's is, for practical purposes, the ratio of their pure variances adjusted (optimally) for pretreatment measurements using analysis of covariance (ANCOVA). When there is no a priori knowledge of target organ, or of a specific parameter's potential for change in response to experimental treatment, a weighted average of parameter variances can be used to estimate sample size requirements. The correlation coefficient between a parameter's posttreatment and pretreatment value should be used to assign weights, and the geometrical mean should be calculated. When the variance by cardiac puncture sample site is employed, the weighted geometrical mean of the sample size ratio of ferret to dog (based on the author's data) can be calculated to be 1.8, or approximately two ferrets for each beagle dog. When the variance and correlation coefficient calculations incorporate the effect of sample site, the weighted geometrical mean of the sample size of ferret to dog is reduced to 1.3, or approximately three ferrets for every two dogs. Table 7.13 and Figure 7.3 illustrate the calculated variance ratios (ferret/dog) for serum chemistry and the various liver enzymes by different sample sizes and sampling sites. These data demonstrate that when a single ferret is used for each dog, the variance ratio for alkaline phosphatase, for example, is approximately 2.5; that is, it can be concluded

Table 7.13 **Effect of Sample Size and Sample Method on the Adjusted (ANCOVA) Variance Ratios for Serum Chemistry Parameters of Ferrets and Dogs**

| | Variance Ratio (Ferret/Dog) | | | |
| | Cardiac Puncture | | Orbital Plexus | |
Parameter	**1:1**	**2:1**	**1:1**	**2:1**
Calcium	1.353	0.677	0.735	0.368
Glucose	4.272	2.136	—	—
BUN	2.777	1.389	—	—
Uric acid	0.640	0.320	0.419	0.210
Bilirubin	0.614	0.307	0.389	0.195
Creatinine	0.802	0.401	—	—
Total protein	1.864	0.932	0.568	0.284
Albumin	2.492	1.246	1.079	0.540
Globulin	1.091	0.546	0.631	0.316
A/G ratio	0.899	0.450	0.749	0.375
Alkaline phosphatase	2.514	1.257	0.760	0.380
LDH	1.647	0.824	1.070	0.535
SGOT	4.244	2.122	3.395	1.698
SGPT	3.992	1.996	2.016	1.008

Note: Missing values show no improvement with orbital plexus collection.
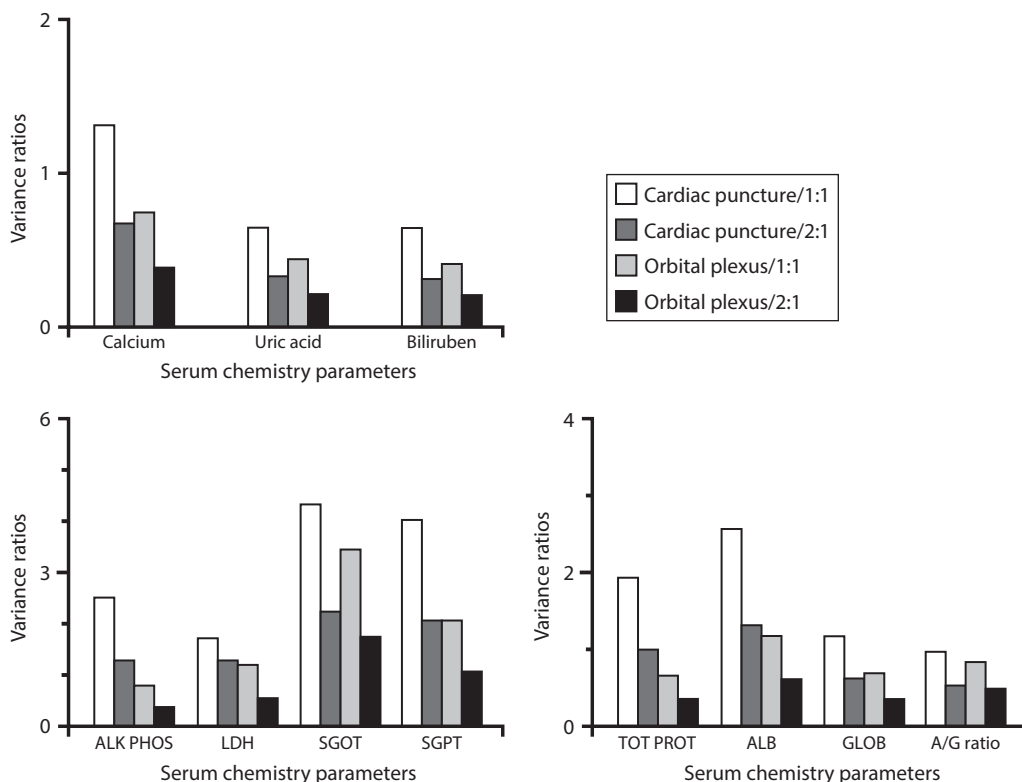
Figure 7.3 Serum chemistry parameter variance ratios for ferret relative to beagle dogs (ferret/dog) as a function of sample site (cardiac puncture or orbital plexus) and sample size (ferret/dog = 1:1 or 2:1). Ratios less than 1 favor the ferret.

that 2.5 times more ferrets are necessary to achieve a parameter variance that is approximately equal to that of the dog. Conversely, when alkaline phosphatase is calculated from an orbital plexus blood sample, the variance ratio drops to approximately 0.75; that is, it is now concluded that 1.25 dogs are necessary to achieve a parameter variance that is approximately equal to that of the ferret.

Urinalysis

Urinalysis is a good general screening procedure for ferrets that can provide a wide variety of useful clinical information regarding an individual ferret's kidneys and the systemic disease that might affect this excretory organ. Collection procedures are similar to what would be performed for rodents, with a special emphasis placed on minimizing fecal contamination of the sample. Prestudy samples will help to identify pathological conditions that would justify eliminating an animal from consideration for testing. Additionally, prestudy urine samples can be subtracted from posttreatment samples to more precisely evaluate the response to experimental treatment.

Thornton et al. (1979) published the means and ranges for volume (mL), sodium (mmol), potassium (mmol), and chloride (mmol) for "feces-free" urine samples collected over a 24 hour period from 40 male and 24 female ferrets. In addition, they analyzed the urine samples for protein, ketones, blood, and bilirubin using reagent strips (Ames Bililabstix) and discussed the results of these findings.

The following database of macroscopic and microscopic urine parameters of the ferret was compiled in our laboratory from ferrets used in the GLP studies discussed previously. All of the ferrets

were obtained from the same supplier (Marshall Farms, North Rose, New York) and were approximately the same age. Animals were placed in rodent metabolism cages for overnight urine collection. The particular type of metabolism cage employed minimized (but probably did not totally eliminate) urine and feces contact and precluded drinking water dilution of the urine samples.

Statistical Treatment of Urine Data

Analysis of variance ($\alpha = 0.05$) techniques were used in the following macroscopic and microscopic urine parameter databases to test for a sex effect for the continuous parameters (specific gravity and total volume). Sex differences were detected for total volume. Therefore, reference values must be considered separately for males and females for this parameter.

Scores were assigned to the responses for the categorical parameters. These scores are summarized in Table 7.14. The assignment of scores was necessary to test for a sex effect and for calculating summary statistics. Table 7.14 must be used to interpret the categorical parameters in Tables 7.15 and 7.16. The scoring method of Grizzle et al. (1969) was applied to test for a sex effect ($\alpha = 0.05$). Sex differences were detected with respect to pH, blood, epithelial cells, WBCs, RBCs, and bacteria. Therefore, reference values must be considered separately for males and females for these parameters. The parameters of urobilinogen, glucose, ketone, and crystals were constant.

The references were calculated as statistical TLs (90% confidence for 95% of the ferret population). These are expressed as a low and high value and are interpreted as follows: 95% of normal ferrets will have a parameter response between the low value and high value (with 90% confidence).

Nonparametric tolerance intervals were calculated for the categorical parameters. Categorical parameters are not normally distributed. The lower and upper limits for nonparametric intervals are of the form of "order statistics" of the categorical data. Examples of order statistics are lowest value, highest value, third highest value, or sixth highest value. The selection of the order statistic depends on the sample size, the confidence level, and the proportion of the population that the interval is to include.

TLs based on the normal distribution were calculated for the continuous, normally distributed parameters. The lower and upper limits are of the form mean $\pm$ factor ∞ standard deviation. The selection of the factor depends on the sample size, the confidence level, and the proportion of the population that the interval is to include.

If a continuous parameter was skewed, an appropriate transformation was determined to normalize the distribution. The normal TLs were then calculated for the normalized data. These limits

Table 7.14   Scores Assigned to Categorical Responses in Ferret Urinalysis

Parameter	Assigned Score				
	0	**1**	**2**	**3**	**4**
Color	Pale yellow	Yellow	Light amber	Amber	Brown
Turbidity	Clear	Slightly cloudy	Cloudy	Turbid	—
Protein, glucose, ketone, blood	Negative	Trace	1+	2+	3+
Bilirubin	Negative	Positive	1+	2+	—
Mucous, epithelial cells, crystals, triple PO4, Ca oxalate, bacteria, sperm	None	Rare	Few	Moderate	Many
Casts	None	Rare	1–4	5–9	—

Assigned Scores for WBC and RBC									
0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
None	Rare	1–4	5–9	10–14	15–19	20–29	30–49	50+	TNTC

Note: TNTC, too numerous to count.

Table 7.15   Reference Semiquantitative Macroscopic Urine Profiles of Adult Male and Female Ferrets Maintained under Controlled Environmental Conditions

Parameter	Sex	*M*	*SD*	N	Min	Max	CT	TL Lower	TL Upper	Method	Sex Effect	Transformation
Volume (mL)	F	21.4	11.9	94	1	59	19.0	4.0	58.0	P	$p < .05$	Ln(X + 5.27)
	M	31.8	21.0	98	6	114	27.4	6.1	88.5			
Color	F	1.0	0.4	94	0	3	1	0	3	P	ns	None
	M	1.0	0.4	98	0	4	1	0	2			
Turbidity	F	1.2	1.1	84	0	3		0	3	P	ns	None
	M	1.2	0.9	98	0	3	1	0	3			
Specific gravity	F	1.043	0.016	94	1.013	1.080	1.042	1.007	1.078	P	ns	None
	M	1.047	0.016	98	1.013	1.084	1.047	1.012	1.082			
pH	F	6.2	0.3	94	6.0	7.5	6.0	6.0	7.5	P	$p < .05$	None
	M	6.1	0.2	98	6.0	7.0	6.0	6.0	6.5			
Protein	F	1.0	1.0	94	0	4	1	0	3	P	ns	None
	M	0.9	0.9	98	0	4	1	0	3			
Glucose	F	0	—	94	0	0	0	0	0	P	ns	None
	M	0	—	98	0	0	0	0				
Ketone	F	0	—	94	0	0	0	0	0	P	ns	None
	M	0	—	98	0	0	0	0				
Bilirubin	F	0.1	0.4	94	0	3	0	0	2	P	ns	None
	M	0.1	0.4	98	0	2	0	0	2			
Blood	F	1.2	1.3	94	0	4	1	0	4	P	$p < .05$	None
	M	0.3	0.7	98	0	4	0	0	3			
Urobilinogen	F	0.1	—	94	0.1	0.1	0.1	0.1	0.1	P	ns	None
	M	0.1	—	98	0.1	0.1	0.1	0.1	0.1			

Note: P, parametric; ns, not significant; Ln, natural logarithm. The CT for urine volume: males = 27.4 mL; females = 19.0 mL.

and the mean of the normalized data were then expressed back in the original units. For example, the transformation for total urine volume (X) was found to be Ln(X + 5.27). The mean, standard deviation, and normal TL for total urine volume of male ferrets, for example, was determined to be $M = 3.48553$, $SD = 0.485852$, lower TL (2.430259), and upper TL (4.540801). To express these in the original units of total volume, the inverse of Ln(X + 5.27) is applied. The inverse is to exponentiate and then subtract 5.27. The resulting CT and low and high limits for male ferret total urine volume are CT (27.4), low (6.1), and high (88.5). The standard deviation of the normalized data cannot be expressed in the original units of total volume meaningfully.

Semiquantitative Macroscopic Urine Parameters

Thornton et al. (1979) reported that the mean 24 hours urine volume and range in volume was greatest in female ferrets ($F = 28$ mL, range = 8–140 mL) as opposed to males ($M = 26$ mL, range = 8–48 mL). In contrast, data listed in Table 7.15 suggest that the opposite is true for overnight urine collection in ferrets and that the distribution is skewed. Collection vessels employed during urine collection should be able to contain approximately 150 mL.

The color of normal ferret urine collected by ureter catheterization varies widely from colorless to deep yellow (unpublished observation from the author's laboratory). However, when metabolism cages are employed, the color can be altered by fecal chromogen contamination. Animals consuming Ralston Purina Ferret Chow 5280, for example, produce a semisoft, dark-green-colored stool that is difficult to separate completely from urine and results in a yellow-green appearance.

Table 7.16 Reference Microscopic Urine Profiles of Adult Male and Female Ferrets Maintained under Controlled Environmental Conditions

Parameter	Sex	*M*	*SD*	N	Min	Max	CT	TL Lower	Upper	Method	Sex Effect	Transformation
Mucous	F	1.3	1.3	94	0	4	2	0	4	P	ns	None
	M	1.5	1.3	98	0	4	2	0	4			
Epithelial cells	F	1.7	1.2	94	0	4	2	0	4	P	$p < .05$	None
	M	2.5	1.1	98	0	4	2	0	4			
Crystals	F	0	—	94	0	0	0	0	0	P	ns	None
	M	0	—	98	0	0	0	0	0			
Triple PO4	F	0.2	0.8	94	0	4	0	0	4	P	ns	None
	M	0.1	0.4	98	0	3	0	0	2			
Ca oxalate	F	0.02	0.2	94	0	2	0	0	0	P	ns	None
	M	0.1	0.5	98	0	4	0	0	2			
Casts	F	0.1	0.5	94	0	3	0	0	2	P	ns	None
	M	0.2	0.6	98	0	2	0	0	2			
WBC	F	3.5	2.4	94	0	9	3	0	8	P	$p < .05$	None
	M	1.3	1.2	98	0	4	2	0	4			
RBC	F	1.7	1.6	94	0	8	2	0	6	P	$p < .05$	None
	M	1.0	1.3	98	0	6	1	0	5			
Bacteria	F	2.1	1.3	94	0	4	2	0	4	P	$p < .05$	None
	M	1.1	1.2	98	0	4	0	0	4			
Sperm	M	1.2	1.5	98	0	4	0	0	4	NA	—	—

Note: P, parametric; ns, not significant.

The fecal pellets, food pellets, and the greenish-tinged urine samples will all give a maximum positive response for blood, protein, and bilirubin with the Ames Multistix test strip. Consequently, when these parameters are key issues of a protocol, urine samples should probably be taken directly from the bladder by catheterization at or before necropsy.

Thornton et al. (1979) reported ketones in 50% of male urines, proteinuria in the majority of animals, and blood in larger amounts in females (attributed to estrus) than in males. In the present database, no ketones were detected in either males or females with the Ames Multistix. However, bilirubin was detected in some samples collected by metabolism cage (confirmed by bladder catheterization), and positive results were found for blood and protein in a number of animals. The presence of urine blood was significantly greater in females than in males and may, as indicated earlier, be associated with estrus. Alternatively, the propensity for urine to be contaminated with feces would seem greater in female ferrets because of their shorter anal–genital space.

Similar to the evaluation of different blood sampling sites, macroscopic urine data collected for ferrets suggest that a comparative study is warranted. This evaluation should include parameter values (by sex) obtained from bladder specimens, immediate versus delayed analysis, the contribution of various diets, and a comparison of estrous and anestrous females.

Reference Microscopic Urine Profiles

Significantly greater amounts of leukocytes, erythrocytes, and bacteria are observed in overnight urine samples of female ferrets when compared with males. However, the levels reported in Table 7.16 are probably well below those that can be considered clinically important in this or any other species. Conn (1963) has indicated that normal human urine, for example, can contain a large range in leukocytes (0–650,000/24 hours) and erythrocytes (0–130,000/24 hours) when measured

by the Addis count. Moreover, the presence of bacteria (in association with WBQ in the overnight urine samples described in Table 7.16) is probably related more to storage conditions and time than to a manifestation of pyuria.

The mean number of RBCs observed in female ferrets corresponds to a Table 7.14 classification range of between rare and 1–4 (per magnification field). To evaluate the contribution of estrus to this measurement, however, catheterization of the bladder would be required.

Organ Weights and Histology for Assessment of Toxicity

Prolonged toxicity testing (as opposed to acute testing) frequently involves the evaluation of all animals (or at least high-dose animals) not only for gross pathological and histological effects at least at the end of the experiment but also for moribund animals sacrificed prematurely. The weights of various organs are usually included in this evaluation.

Organ Weights and Transformations

The absolute organ weights of the ferret (Table 7.17), when divided by the body weight at necropsy, are routinely expressed as relative organ weights (Table 7.18). This calculation assumes, however, that the organ weight increases in proportion to the body weight. This assumption is approximately valid for the liver weight and kidney weight but (as can be demonstrated in most species) is grossly violated for the brain weight. Consequently, a revised relative organ weight must be calculated (Table 7.19) such that the calculation becomes independent of the body weight. Figure 7.4 demonstrates how ferret brain weight, for example, varies with respect to sex and body weight and is then made proportional to body weight when the appropriate transformation is applied.

A species comparison of revised relative organ weights is listed in Table 7.20. This particular control animal database includes 94 male and 87 female rats, 24 male and 24 female beagle dogs, and 48 male and 48 female ferrets. Table 7.20 demonstrates that (because the exponents are all less than 1) the ratio of organ weight (especially the brain) to body weight (unrevised) would be larger

Table 7.17   Reference Absolute Organ Weights (g) of Adult Male and Female Ferrets Maintained under Controlled Environmental Conditions

Organ	Sex	*M*	*SD*	N	Min	Max	CT	TL Lower	TL Upper	Method	Sex Effect	Transformation
Brain	F	5.95	0.41	48	4.57	6.83	5.95	5.00	6.90	P	$p < .001$	None
	M	7.35	0.62	48	5.70	8.94	7.34	5.92	8.76			
Heart	F	4.02	0.52	48	3.01	5.80	4.04	2.84	5.64	P	$p < .001$	None
	M	6.62	0.81	48	3.88	8.25	6.62	4.76	8.48			
Lung	F	5.70	0.78	48	4.03	7.57	5.71	3.89	7.53	P	$p < .001$	None
	M	9.08	1.09	48	6.61	13.03	9.11	6.64	11.58			
Liver	F	21.9	4.5	48	12.5	35.8	22.1	12.1	32.2	P	$p < .001$	None
	M	37.8	5.0	48	19.7	44.8	37.8	26.4	49.2			
Spleen	F	4.73	1.63	48	1.99	8.62	4.77	1.03	8.50	P	$p < .001$	None
	M	7.92	1.73	48	3.26	12.06	7.92	3.92	11.91			
Left kidney	F	2.07	0.27	48	1.58	2.84	2.08	1.47	2.69	P	$p < .001$	None
	M	3.40	0.37	48	2.45	4.24	3.40	2.55	4.25			
Right kidney	F	1.99	0.27	48	1.46	2.62	2.00	1.38	2.63	P	$p < .001$	None
	M	3.27	0.36	48	2.26	4.23	3.27	2.44	4.10			

Note: P, parametric.

Table 7.18 Reference Relative Organ Weights (g/kg) of Adult Male and Female Ferrets Maintained under Controlled Environmental Conditions

Organ	Sex	*M*	*SD*	N	Min	Max	CT	TL Lower	TL Upper	Method	Sex Effect	Transformation
Brain	F	7.03	1.04	48	4.34	10.12	7.09	4.86	9.31	P	p < .001	g/kg
	M	4.75	1.21	48	3.39	11.60	4.60	3.06	6.15			
Heart	F	4.73	0.68	48	2.42	6.03	4.78	3.41	6.15	P	p < .001	g/kg
	M	4.19	0.49	48	3.51	5.78	4.19	3.05	5.33			
Lung	F	6.68	0.91	48	3.83	8.49	6.74	4.86	8.63	P	p < .001	g/kg
	M	5.80	1.10	48	4.27	10.81	5.69	3.80	7.58			
Liver	F	25.6	4.3	48	10.4	36.3	25.9	17.4	34.5	P	p < .001	g/kg
	M	23.9	2.6	48	19.5	30.3	23.9	17.9	29.8			
Spleen	F	5.56	1.92	48	2.34	10.1	5.61	1.21	10.0	P	p < .001	g/kg
	M	5.01	1.09	48	2.06	7.63	5.01	2.48	7.54			
Left kidney	F	2.44	0.35	48	1.30	3.32	2.46	1.74	3.18	P	p < .001	g/kg
	M	2.18	0.38	48	1.57	3.54	2.18	1.29	3.06			
Right kidney	F	2.34	0.34	48	1.19	3.14	2.37	1.68	3.06	P	p < .001	g/kg
	M	2.09	0.35	48	1.65	3.27	2.09	1.28	2.89			

Note: P, parametric.

Table 7.19 Reference Relative Organ Weights (g/kg) of Ferrets (Sexes Combined) Maintained under Controlled Environmental Conditions

Organ	Sex	*M*	*SD*	N	Min	Max	CT	TL Lower	TL Upper	Method	Sex Effect	Transformation
Brain	M + F	6.35	0.58	96	4.65	8.90	6.33	5.22	7.45	P	ns	$g/kg^{0.28}$
Heart	M + F	4.61	0.52	96	2.61	6.32	4.63	3.59	5.67	P	ns	$g/kg^{0.75}$
Lung	M + F	6.52	0.87	96	4.25	9.54	6.52	4.81	8.22	P	ns	$g/kg^{0.66}$
Liver	M + F	25.3	3.5	96	10.9	36.2	25.5	18.6	32.3	P	ns	$g/kg^{0.83}$
Spleen	M + F	5.40	1.45	96	2.36	9.66	5.44	2.34	8.53	P	ns	$g/kg^{0.79}$
Left kidney	M + F	2.41	0.32	96	1.44	3.15	2.42	1.75	3.08	P	p < .05	$g/kg^{0.68}$
Right kidney	M + F	2.31	0.30	96	1.31	3.05	2.32	1.69	2.95	P	p < .05	$g/kg^{0.68}$

Note: P, parametric; ns, not significant.

for smaller animals than for larger animals. The revised organ weight formulas remove the effect of body weight entirely and provide a valuable addition to the statistical protocol.

Common Histological Findings in Ferret Tissues

The histological findings detailed in Table 7.21 provide a summary of what has been reported by two independent pathologists for 40 UT control animals and 64 intravenously saline-treated (ST) control animals. Most noteworthy in this database is the fact that what one pathologist might consider reportable another pathologist might consider normal for the species (and thus not reportable). This is evident by the contrasting frequency of histological citations for specific organs, with extramedullary hematopoiesis of the spleen being the most extreme example.

Mononuclear cell infiltration is apparently the most frequently observed histological finding in ferret tissues. This particular microscopic finding occurred in 100% of the livers examined and to a lesser and more variable degree in various other organs. The mean severity grade of slight assigned (by both pathologists) to the degree of mononuclear cell infiltration of the liver implies
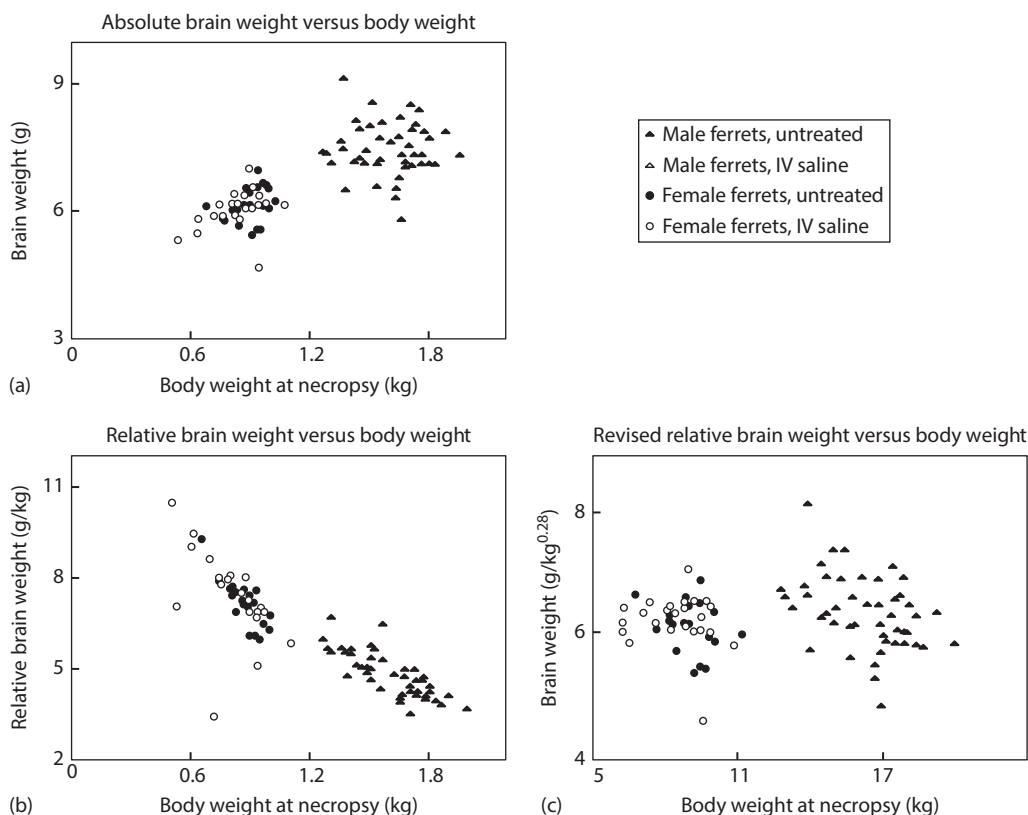
Figure 7.4 Scattergram of ferret brain weight expressed as the absolute weight (a), relative weight (b), and revised relative weight (c). By applying the power transformation to body weight (i.e., revised relative organ weight), the effect of sexual dimorphism is eliminated; thus, sample size (N) is increased accordingly.

Table 7.20   Revised Relative Organ Weight for Rats, Beagle Dogs, and Ferrets (Revised Relative Organ Weight = Organ Weight/[Body Weight]^{power})

Organ	Power Factor (Exponent)		
	Rat	**Beagle Dog**	**Ferret**
Brain	0.17	0.18	0.28
Lung	0.44	0.71	0.66
Heart	0.72	0.81	0.75
Liver	0.82	0.95	0.83
Spleen	0.40	0.97	0.79
Kidney	0.76	0.94	0.68

that moderate or greater infiltrations could have occurred in approximately one-third of the animals examined. Therefore, to minimize the number of moderate infiltrations and decrease the overall mean score, McLain and Lin (1989) devised an index method of severity prediction based on pre- and posttreatment blood samples (see later section). The use of this index to predict the degree of lymphocyte infiltration enables the culling of animals with unacceptable scores (defined by the

Table 7.21 Microscopic Findings of the IV ST and UT Male and Female Ferret's Major Organs as Reported by Two Independent Pathologists (Incidence [%], Severity[a])

	Pathologist		
Organ/Microscopic Finding	A(ST)	B(ST)	B(UT)
Brain			
Mononuclear cell infiltration, choroids plexus	15/32	0/32	0/40
	(46.9)	(0.0)	(0.0)
	NG	—	—
Mononuclear cell infiltration, meninges	2/32	0/32	0/40
	(6.3)	(0.0)	(0.0)
	1.0	—	—
Lungs			
Pneumonitis	26/32	26/32	33/40
	(81.3)	(81.3)	(82.5)
	1.7	1.5	1.5
Heart			
Fibrosis	0/32	4/32	5/40
	(0.0)	(12.5)	(12.5)
	—	1.2	1.2
Liver			
Mononuclear cell infiltration, portal or periportal	32/32	32/32	40/40
	(100)	(100)	(100)
	2.0	2.0	2.2
Kidney			
Mononuclear cell infiltration, interstitial	3/32	0/32	0/40
	(9.4)	(0.0)	(0.0)
	1.3	—	—
Nephritis, glomerulo- or interstitial	1/32	13/32	13/40
	(3.1)	(40.6)	(32.5)
	1.0	1.2	1.2
Spleen			
Extramedullary hematopoiesis	0/32	32/32	40/40
	(0.0)	(100)	(100)
	—	2.5	2.4
Stomach			
Mononuclear cell infiltration	1/32	1/32	2/40
	(3.1)	(3.1)	(5.0)
	3.0	2.0	1.5

[a] Severity: 1, minimal; 2, slight; 3, moderate; 4, moderately severe/high; 5, severe/high; NG, not graded.

investigator). Alternatively, the index can be used as a blocking factor to randomize animals into test groups such that lymphocyte infiltration scores between groups are similar.

Although the severity is slightly greater, the incidence of pneumonitis reported in Table 7.21 for ferrets is not markedly different from what has been observed for beagle dogs (unpublished observations). On the other hand, the minimal to slight cardiac fibrosis observed, which is apparently a residual effect of the blood sampling procedure, is not a common finding in the beagle dog. The pathology section of this chapter provides a more detailed evaluation and overview of the toxicological histopathology of the ferret.

Derivation and Application of the LvLI

When using ferrets as a nonrodent animal model for safety assessment studies, the preexisting histological lesions of the species must be addressed. Obviously, improved hygiene and breeding efforts by the vendor will reduce the frequency of some lesions. Preceding this, however, statistical control can be used to accommodate or minimize their effects.

To control existing liver lesions common to the ferret, a database was constructed from pre- and posttreatment hematology and clinical chemistry measurements obtained by cardiac puncture from 152 male and 152 female ferrets. Tissues from all animals were evaluated microscopically for the presence and severity of lymphocyte infiltration in various organs. Organs evaluated included (but were not limited to) the liver, lung, kidney, thyroid, stomach, colon, and brain. The histology scoring system used was 0, none; 1, minimal; 2, slight; 3, moderate; 4, high; and 5, severe. Attempts were made to predict the pathologist's score for liver lymphocyte infiltration, for example, by quantitative lesion indexes based on (1) pretreatment clinical chemistry and hematology measurements of individual animals (pretreatment lesion index) and (2) all pre- and posttreatment measurements (including organ weights) of these same animals (final lesion index).

The benefits of using pretreatment lesion indexes for prediction of lesion severity and presence include the following: (1) Because an animal's organ tissue cannot be examined until the animal is sacrificed, the index can be used to screen for healthy animals based on measurements of their pretreatment blood samples. (2) The index can be used as a blocking factor to randomize animals such that all treatment groups (including the control group) contain animals with similar lesions. (3) The index can be used as a covariable in the statistical analysis to control for variation due to preexisting lesions. Similarly, the benefits of using the final index include the fact that it is an objective, quantitative, and continuous score instead of a subjective and ordinal score (pathologist score). In addition, it is statistically more powerful and easier to evaluate when comparing organ lesions across treatment groups.

The lesion index is the linear combination of all parameters that yield the best geometric separation between "less than moderate" and "moderate or higher" lymphocyte infiltration conditions. In other words, it is the weighted average of all parameters that yield the most power (or the least error) to predict a moderate or higher lymphocyte infiltration of an animal. The weight (coefficient) that is assigned to each parameter depends on how much that parameter contributes to the prediction power.

Table 7.22 lists the liver lymphocyte infiltrate index based on pretreatment parameters (LvLI-pre). The first column presents all parameters listed in order of decreasing importance of their contribution in prediction power. The third column lists the coefficient (weight) that was assigned to each parameter. The fourth column shows how each parameter correlated with the index. For this index, globulin is the strongest predictor, with a correlation coefficient of 0.583. After knowing the globulin level, the lymphocyte count provides more additional predictive power than any other parameter. Similarly, MCHC is the third highest predictor after we know the globulin and lymphocyte count. Total protein has a higher correlation (0.478) with the index than does MCHC in this example, but it is given a lower priority because it is highly correlated with globulin. In other words, the joint information from globulin, lymphocyte count, and MCHC is more predictive than the joint information from globulin, lymphocyte count, and total protein. The same argument can be used for the rest of the information in Table 7.22.

Table 7.23 lists the LvLI based on all information collected, including the organ weights at necropsy and all pretreatment data. The LvLI-pre explains 72% (0.846) of this index. The posttreatment SGPT is a strong predictor. The posttreatment globulin is a weak predictor because it is highly correlated with LvLI-pre.

Table 7.22   Prestudy Parameters Used for the Calculation of a Liver Lymphocyte Infiltrate Index

$$\left(\text{LvLI} - \text{pre} = 6.2335 + \sum_{I=1}^{18} C_I Y_I\right)$$

Parameter (X)[a]	Transformation (Y)	Weight Coefficient (C)	Correlation Coefficient (Rank)[b]
Globulin	Ln(X − 1.23)	1.11690	0.583 (1)
Lymphocyte count	Ln(X)	0.78835	0.476 (3)
MCHC	X	0.25618	0.218 (7)
LDH	Ln(X − 120)	−0.47096	−0.311 (5)
SGPT	Ln(X − 40)	0.88691	0.361 (4)
Uric acid	Ln(X + 0.74)	−0.94505	−0.093 (13)
RBC	X	0.30556	−0.225 (6)
BASO count	Ln(X + 0.001)	0.13193	0.156 (10)
Alkaline phosphatase	Ln(X − 10)	−0.64420	−0.055 (17)
Cholesterol	X	0.01485	0.092 (14)
Glucose	Ln(X + 96)	−1.83530	−0.158 (9)
MONO count	Ln(X + 0.001)	0.06817	0.178 (8)
STAB count	Ln(X + 0.001)	−0.11888	−0.088 (15)
Albumin	X	−0.85864	−0.029 (18)
Total protein	X	0.41570	0.478 (2)
BUN	Ln(X − 9)	−0.44574	−0.106 (11)
Creatinine	X	1.80620	0.095 (12)
Fibrinogen	Ln(X − 63)	−0.21236	−0.056 (16)

[a] Parameters are listed in order (top to bottom) of additional predicting power.
[b] The correlation coefficient explains the predicting power of the parameter if all other parameters are unknown.

Success Rate of LvLI-pre and LvLI

Table 7.24 shows the success rate of LvLI-pre and LvLI. For those ferrets with LvLI-pre greater or equal to 3, we would expect a moderate or high severity score (degree of lymphocyte infiltration) when their liver tissue is evaluated by the pathologist at sacrifice (2–3 weeks later). Among the 74 ferrets in the present database that were judged to have a moderate or high severity score by the pathologist, 65 were judged to have moderate or high scores with the LvLI-pre. Therefore, we have 88% confidence that the index (based on pretreatment blood samples) would agree with the pathologist's opinion 2–3 weeks later. Similarly, among the 224 ferrets that were judged to have a slight or less severity score by the pathologist, 196 (88%) were judged the same by the LvLI-pre.

LvLI is shown to have a 92% (68/74) success rate in predicting moderate or high liver lymphocyte infiltrate severity scores and has a 96% (212/221) success rate in predicting slight or moderate scores as judged by a pathologist.

Typical Protocols

Acute Toxicity Testing

Traditionally, the single test that is conducted on essentially all chemicals that are of any biological interest is the acute toxicity test. In the classical sense, the test consists of administering the compound to the animals on one occasion for a time period that is usually less than 24 hours.

Table 7.23   Poststudy Parameters Used for the Calculation of a Liver Lymphocyte Infiltrate Index

$$\left(LvLI = 8.5207 + \sum_{I=1}^{21} C_I Y_I \right)$$

Parameter (X)[a]	Transformation (Y)	Weight Coefficient (C)	Correlation Coefficient (Rank)[b]
LvLI-pre	X	0.44589	0.846 (1)
SGPT	Ln(X − 40)	0.72639	0.630 (2)
A/G ratio	X^2	0.06011	−0.409 (5)
Creatinine	X	0.85106	0.192 (11)
Potassium	Ln(X − 3.5)	−0.49440	−0.196 (10)
Heart, rev.-rel.	X	0.38091	0.096 (15)
Kidney, rev.-rel.	X	−0.47868	0.004 (21)
Spleen, rev.-rel.	X	0.08443	0.388 (6)
A/G ratio-pre	X^2	0.15299	−0.381 (7)
MCV	X	−0.07150	−0.081 (16)
Glucose	Ln(X + 96)	−1.05990	−0.151 (12)
HCT-pre	X	−0.03010	−0.226 (9)
STAB count-pre	Ln(X + 0.001)	−0.06265	−0.081 (17)
Phosphorus-pre	X	0.09743	0.138 (13)
Lung, rev.-rel.	X	−0.05949	−0.044 (19)
Cholesterol-pre	X	0.00204	0.076 (18)
EOS count	Ln(X + 0.001)	−1.05796	0.008 (20)
Liver, rev.-rel.	X	0.01964	0.356 (8)
BASO count-pre	Ln(X + 0.001)	0.02406	0.126 (14)
Globulin	Ln(X − 1.23)	1.48610	0.551 (3)
Total protein	X	−0.51067	0.479 (4)

[a] Parameters are listed in order (top to bottom) of additional predicting power.
[b] The correlation coefficient explains the predicting power of the parameter if all other parameters are unknown.

Table 7.24   Performance Measurement of LvLI-pre and LvLI

	Scored by Pathologist	
	Slight or Less	Moderate or High
Based on LvLI-pre		
Slight or less, LvLI < 3	196	9
Moderate or high, LvLI > 3	28	65
Total	224	74
Based on LvLI (final)		
Slight or less, LvLI < 3	212	6
Moderate or high, LvLI > 3	9	68
Total	221	74

The purpose of the test is to determine the symptomology consequent to administration of the compound and, as it is used less frequently today, to determine the order of lethality of the compound. Essentially, all initial acute toxicity tests are performed in rodents because of their small size, availability, and the abundance of reference toxicological data generated for the species. Subsequent testing using similar procedures is performed in other species such as the ferret for the purpose of contrast and confirmation.

The ferret has proven especially useful in the screening type of acute toxicity study. These studies aid in identifying compounds of such low toxicity that, when considered in relation to a proposed use of low exposure, extensive investigations to make a judgment of safety are not justified. In the screening type of acute toxicity study, the amount of test agent to which an animal is exposed is usually so massive that it generally bears no practical relationship to the expected human exposure.

Acute Oral Testing

Acute oral toxicity testing in ferrets, commonly performed subsequent to rodent testing, is designed to elicit the qualitative and quantitative nature of the toxic effects from a one-time oral exposure to a large dose of a chemical or test agent. Whether the purpose of testing is to provide data for estimating the lethal dose for 50% of a group of animals or for demonstration that some large multiple of the potential human dose does not result in an irreversible manifestation of toxicity, the number of animals employed should be sufficient for a sound statistical evaluation. Typically, six to eight adult ferrets/sex/group are sufficient when there is no a priori knowledge of the raw effects resulting from exposure to the test agent. Procedures for testing would be similar to those prescribed for rodents, with the exception that emesis should be included in the physical and observational examination protocol. In addition, phonation should be monitored as it is more common in the ferret than it is in rodents.

Acute Dermal Testing

The ability of some chemicals to penetrate intact and abraded skin and produce systemic toxicity is well known, and steps should therefore be taken to evaluate this possibility when appropriate. The albino rabbit is, of course, the animal most frequently used in assessing dermal toxicity. However, the mouse, rat, guinea pig, and dog have also been used. The ferret would most likely parallel the dog in this type of evaluation, with its thick skin severalfold less permeable than that of the rat or rabbit (thus closer to human skin).

An ideal dermal application site in the ferret is the dorsal surface just above or at the level of the shoulders. Large "rat jackets" or vests can be fitted to the animals when it is necessary to cover a test material. After an appropriate exposure period, excess material is removed, and the local changes and any gross signs of toxicity are noted. Animals should be observed for an appropriate period of time and postmortem studies performed.

Acute Inhalation Toxicity

Test article exposure by inhalation is probably the most time consuming and expensive of all toxicological dosing procedures. Vinegar et al. (1985) have convincingly argued, however, that the ferret is a less expensive substitute for the dog in acute inhalation toxicity testing. Moreover, because the ferret has more submucosal glands in the bronchial walls and an additional generation of terminal bronchioles, these authors have stated that the ferret lung is closer anatomically to the human lung than is the dog lung (see earlier discussion). Protocols for acute inhalation toxicity in ferrets should parallel those used for rodents, with the possible exception of perhaps increasing the effect sample size to accommodate the increased heterogeneity of the species. In addition, because pulmonary changes are likely manifestations of response to experimental treatment, ferrets selected for study should be thoroughly screened during quarantine for the absence of respiratory disease. Application of an LvLI, as described previously for the liver, can detect ferrets with moderate or higher infiltrates with an 88% success rate when using LvLI-pre and a 94% success rate when all variables are used in the calculation.

Subchronic Toxicity Testing

Subchronic toxicity procedures are designed to determine the adverse effects that might occur during repeated exposure over a period of a few days to usually 3 months (90 days). The subchronic procedures usually include the routes of exposure expected for man, with exposure levels lower than in the acute toxicity protocols. A high exposure level that is judged to be sufficiently large to produce adverse effects and at least one lower exposure level that is not expected to produce adverse effects are used. Intermediate exposure levels should be introduced when they are considered necessary. The number of ferrets used should be sufficient for statistical confidence (usually six to eight adult ferrets/sex/group). Observations should include overt signs of toxicity, food consumption (when appropriate), body weight change, hematology, clinical chemistry, urinalysis, organ weights, and gross and microscopic pathology. To increase the statistical power, clinical pathology should be evaluated as the change from baseline (i.e., pretreatment samples must be collected from all animals).

Clinical observations of test animals should include daily cageside evaluations as well as detailed examinations performed at least once per week. The detailed physical examination can be scheduled with the body weight measurement. Standard procedures employed for other test species are acceptable. Additional clinical notations should include inspections for excessive fur or hair under the cage, swelling of the vulva in females, and testicular prominence in males. To maintain all animals in a sexually inactive state, they should be segregated by sex (when possible) and subjected to shortened photoperiods. Recovery studies can be included if the changes observed indicate that this type of procedure is necessary. As with the other types of toxicity procedures, subchronic toxicity data generated with ferrets are most complementary when expressed as a contrast to subchronic rodent data.

Long-Term Bioassays for Chronic Toxicity and Carcinogenesis

The classic approach to the study of chronic toxicity and the carcinogenic potential of test substances involves studies in two or more species of animals (one of which should ideally be a nonrodent) for periods of time ranging from many months to several years. Rats and mice have, of course, been the primary test species for these protocols. However, long-term testing with ferrets would seem to be a plausible and welcomed alternative.

Irrespective of the variability observed in the normal ferret's clinical or microscopic profile, with increasing dosage in the continuum of the dose–response relationship, a region is generally entered where the effects are clearly adverse.

The detection of adverse effects in chronic toxicity protocols begins with gross observations of the intact animal in terms of growth, appearance, and activity. The next point of discrimination is at the organ-system level, wherein changes of a biochemical and physiological nature are assessed. These are followed by an examination of morphological changes at the gross and cellular levels in sacrificed animals or biopsy material. Clearly, adverse effects that occur in ferrets or any other species are those that result in impairment of functional capacity (as determined by anatomical, physiological, and biochemical or behavioral parameters) or in a decrement of the ability to compensate for additional stress, are irreversible during exposure or following cessation of exposure, and enhance the susceptibility of the individual to the deleterious effects of other environmental influences.

Developmental Toxicity Assessment

It is evident that there is no one animal species that can be considered ideal for evaluating human developmental toxicity. Ferrets are no exception to this dilemma, of course, and provide just a higher level of evaluation for embryo toxicity than what is offered with rodents. The ferret's chief advantage in reproductive toxicity assessment is its small size and the fact that it does not have the atypical yolk-sac placenta that is common to the rat.

Protocols for evaluation of developmental toxicity in the ferret follow guidelines similar to those that would be used for rodents, with the exception that animals obtained from a reputable supplier should be vaccinated for distemper and subjected to a thorough veterinary examination on arrival at the vivarium. Estrous females at their peak of vulval swelling should be placed with sexually active males of proven fertility and observed for successful coitus on several successive occasions. The first successful coitus is considered day 0 of pregnancy because ovulation generally occurs approximately 30 hours postcoitus. The examination of vaginal lavage for motile sperm after observed mating would be helpful in determining successful insemination. Presumed-mated jills can also be shipped from the supplier (Marshall Farms, North Rose, New York) the day following coitus (day 1 of gestation) with no apparent effect on reproductive parameters if transportation is completed on gestation day 1. Acclimatization to the vivarium is easily accomplished between arrival and day 12 of gestation when dosing should begin. Vulval regression and obvious weight gain would indicate successful conception. The critical period in the ferret is generally accepted as days 12–30, with implantation occurring on approximately day 12.

Litters should be taken by cesarean section on gestation day 35 and subjected to the same examination procedures used for rats and rabbits. That is, living fetuses should be weighed, sexed, and examined externally and internally by gross dissection of all cavity organs or by the Wilson or Staples techniques. Skeletal visualization by potassium hydroxide (KOH) clearing should be done on a predetermined number of fetuses. Other parameter evaluations and reporting are similar to what would be done for rodents.

Routine acceptance of the ferret in developmental toxicity studies is still to be established within the various governmental agencies. It is recommended, therefore, that a positive control group (15 mg/kg all-trans retinoic acid on day 14 of gestation) be included with every study.

Summary

Review of Advantages and Disadvantages

The most obvious advantages to the use of the ferret as an animal model in toxicology include their relatively low cost, small body size, ease of handling (mild disposition) and maintenance, and ability to adapt to most existing facilities and laboratory equipment. Less obvious advantages include the current lack of opposition to their use (traditionally voiced by antivivisectionists), and their apparent physiological and biochemical similarities with humans. The increasing popularity of ferrets in biomedical research correlates highly with the growth of the biotechnology industry. In this respect, the ferret requires only about one-tenth of the limited test article that would be needed for the more conventional beagle dog.

Disadvantages of the ferret include their large relative heterogeneity, current lack of extensive databases, lack (temporary) of an approved rabies vaccine, lack of "virus-free" ferrets, limited availability, and the small number of vendors selling the animal.

Steps That Can Be Taken to Minimize Disadvantages

A significant portion of this chapter is devoted to the presentation and discussion of material that could be viewed as a beginning database for the ferret. In addition, novel methods for decreasing the level of heterogeneity in this species (or any other species) and eliminating undesirable animals from testing consideration have been proposed. New information has been presented with respect to the pending approval of a rabies vaccine for ferrets. However, only the continued use of and demand for ferrets will cause the number of vendors available to increase, and only concentrated research and breeding efforts will provide the scientific community with a virus-free ferret.

PATHOLOGY

Shayne Cox Gad

The ferret (*M. putorius furo*) is thought to have originated from the wild European polecat or ferret and might also be related to the steppe polecat. Ferrets share many anatomical, metabolic, and physiologic features with humans, which has promoted their use as an animal model. Ferrets are used in biomedical research in a wide variety of studies including cardiopulmonary, neurological, GI research, and infectious diseases. Ferrets have been used in cardiovascular research to examine ischemia and ion exchange in the heart muscle, pulmonary mucus secretion related to asthma or influenza, neurological changes associated with brain and spinal cord injury, and gastric infections and ulcerations. Ferrets have also been used as a model for the demonstration of medical procedures such as pediatric tracheal intubation.

Infectious and Parasitic Diseases

Shapiro and DeMello (2010) have published a text covering parasites and general pathology methods for a number of laboratory species, including the ferret.

Canine Distemper

This is a morbillivirus disease with essentially 100% fatality. Disease progression ranges from 12 to 42 days. The disease is profoundly immunosuppressive, and surviving animals succumb to neurologic dysfunction. Treatment is not recommended (Hoover et al., 1989; Kaufman et al., 1982). Gross lesions include photophobia, oculonasal discharge, bronchopneumonia, hyperkeratosis of the planum nasale and footpads, and a papular rash beginning on the chin and progressing to a generalized form. Microscopically, there is pneumonitis with syncytia, multifocal dermatitis, hyperkeratosis, and eosinophilic, intracytoplasmic, and intranuclear inclusions in a wide variety of epithelial cells and neurons. A nonsuppurative encephalitis with demyelination can be seen in animals with neurologic disease (Fox et al., 1998a; Williams et al., 1988).

Parvoviral Infection (Aleutian Disease)

Aleutian disease is caused by an antigenically related parvovirus that causes Aleutian disease in mink. In ferrets, the disease is much more insidious with hypergammaglobulinemia, CNS disease, wasting syndrome, proteinuria, and, in late stages of the disease, an immune complex glomerulonephritis (Alexandersen et al., 1994; Ohshima et al., 1978; Oxenham, 1990; Porter et al., 1982; Welchman et al., 1993). Gross lesions observed late in the course of the disease include splenomegaly, lymphadenopathy, petechial hemorrhage, and hematuria. Microscopic findings include prominent plasmacytic infiltrates (plasmacytosis) in the renal interstitium, hepatic portal areas, splenic red pulp, lymph nodes, and the bone marrow. In most cases, there will be marked membranous glomerulonephritis and numerous ectatic protein-filled tubules as a result. Vasculitis can be seen in almost any organ (Alexandersen et al., 1994; Daoust and Hunter, 1978; Palley et al., 1992; Wolfensohn and Lloyd, 1995).

Coronavirus-Associated Epizootic Catarrhal Enteritis and Serious Acute Respiratory Syndrome

Epizootic catarrhal enteritis (ECE) is a diarrheal disease that causes epizootics of high morbidity (up to 100%), but low mortality. The diarrhea is rapidly dehydrating, and most mortalities occur in older animals with concurrent illness. Symptoms include vomiting and passage of a dark-green

stool with abundant mucus. Grossly, the intestines might be flaccid with a moderate amount of watery ingesta. Microscopically, lesions include lymphocytic enteritis, vacuolar degeneration and necrosis of villus enterocytes, villus atrophy, fusion, and blunting (Williams et al., 2000). Serious acute respiratory syndrome (SARS) is a family of respiratory diseases that has become of great concern as a threat to human public health (Van den Brand et al., 2008). The pathology is poorly described. Diffuse alveolar damage is always present.

Rabies

The incidence is low with fewer than 25 confirmed cases since 1954. The disease can result in both furious (less common) and dumb forms and might present as a progressive hind limb paralysis. There are no gross lesions, and microscopically intracytoplasmic eosinophilic viral inclusions (Negri bodies) might be demonstrated on H&E stains or on standard fluorescent antibody tests (Fox et al., 1998b).

Influenza

Ferrets are the only domestic animal species that is susceptible to the human influenza viruses (Kroeze et al., 2012; Renegar, 1992; Smith and Sweet, 1988). For this reason, they are often used as animal models in influenza research and often infected by their human owners. The disease is quite similar to that in humans, with clinical signs being photophobia, a catarrhal nasal discharge, sneezing, coughing, pyrexia, anorexia, and malaise. Gross lesions are generally minimal, with congestion and exudation of the nasal mucosa and mild reddening of the tracheal mucosa. Microscopically, there is mild subacute inflammation and occasional necrosis of the nasal mucosa. A mild subacute interstitial pneumonia might be present (Fox et al., 1998b; Renegar, 1992; Smith and Sweet, 1988). The ferret provides the primary model for pneumonia from seasonal human influenza, a viral infection (Smith et al., 2011; Van den Brand et al., 2012).

Helicobacter mustelae

Helicobacter mustelae–associated gastric disease in ferrets over the age of 4 years resembles *Helicobacter pylori* in man and nonhuman primates (Fox et al., 1990, 1991; Otto et al., 1990). The bacterium causes disease via two mechanisms: (1) the stimulation of a marked lymphoplasmacytic inflammatory response, resulting in loss of glandular epithelium, most prominently in the pylorus and (2) the ability to increase the pH of the stomach (Fox et al., 1990, 1993; Gottfried et al., 1990). Gross lesions in advanced cases might be limited to gastric ulcers. Microscopically, gastritis is accompanied by the presence of bacteria in the superficial mucous or within the gastric glands, and these can be demonstrated with Warthin–Starry stain (Fox et al., 1990, 1991).

Proliferative Colitis

This is an uncommon, sporadic disease that is usually seen in young male ferrets under 1 year of age affecting only one or two animals in a large colony (Finkler, 1992; Fox et al., 1982). Clinical signs include tenesmus and frequent painful defecation with frank blood and mucus. The disease is caused by an intracellular campylobacter-like organism bacteria belonging to *Ileobacter* (*Desulfovibrio*) sp. (Fox et al., 1988, 1989, 1994). Grossly, there is marked thickening of the colonic wall, and the mucosa has a cobblestone appearance. Microscopically, the mucosa is multifocally thickened due to proliferation of immature epithelial cells and mixed inflammatory cell infiltrate. Bacteria in the apical cytoplasm of epithelial cells can be demonstrated with Warthin–Starry stain (Finkler, 1992; Fox et al., 1994; Krueger et al., 1989).

Clostridium perfringens

Gastroenteritis associated with *Clostridium perfringens* type A has been reported in black-footed ferret kits. Grossly, there is gastric bloat and multifocal intestinal hemorrhage. Microscopically, there is marked coagulative necrosis of the intestinal mucosa with numerous adherent bacilli. In addition to perfringens, botulism due to toxins produced has been reported (Schulman et al., 1993).

Tuberculosis

Ferrets are susceptible to human, bovine, and avian mycobacteria. Mycobacterium avium-intracellulare infection is a rare condition in ferrets that is most commonly seen in the GI tract and mesenteric lymph nodes. Mesenteric lymphadenopathy is the most common gross lesion. Microscopically, there is lymphadenitis with large foamy macrophages containing acid-fast bacilli (Schultheiss and Dolginow, 1994).

Mastitis

Pregnant jills in the first few weeks of lactation can develop mastitis. Hemolytic *E. coli* is the most commonly isolated organism and results in a syndrome of gangrenous mastitis. If UT, jills rapidly become septic or endotoxemic. *Staphylococcus aureus* is occasionally cultured from cases of mastitis and produces a more suppurative, less necrotic form of mastitis. Grossly, affected teats are swollen, necrotic, black, firm, and nonpainful. In *S. aureus* mastitis, the mammary glands are hot, painful, and reddish in color; purulent exudate might be expressed from the lactiferous ducts. Microscopically, the primary lesion in *E. coli* mastitis is diffuse severe coagulative necrosis, hemorrhage, and edema that extends into the adjacent adipose tissue and muscle. In staphylococcal mastitis, there is less evidence of infarction. Purulent galactophoritis and mastitis are present. Bacteria might be present in the tissues in both cases (Fox et al., 1998b; Liberson et al., 1983).

Intestinal Parasites

With the exception of coccidia, intestinal parasites are uncommon in ferrets. *Cryptosporidium* sp. infection can be prevalent in a colony, but it does not appear to be very pathogenic (Rehg et al., 1988). Three species of coccidia have been seen in ferrets: *Eimeria furo*, *E. ictidea*, and *Isospora laidlawii*. Although most coccidial infections are subclinical, lethal coccidial infections are occasionally seen in young kits. Grossly, digested blood might be present in the GI tract of several affected kits. Microscopically, numbers of parasites range from very low to extremely high in severe infections, and all stages of the parasite, including micro- and macrogametocytes, can be seen (Blankenship-Paris et al., 1993).

Dirofilariasis

Canine heartworm infection is uncommon, due to the fact that most ferrets are kept indoors. Due to the small size of the ferret heart, as few as two heartworms can result in fatal cardiac insufficiency (Miller and Merton, 1982; Moreland et al., 1986; Parrott et al., 1984). Grossly, the presence of heartworms within the right ventricles and pulmonary artery can be construed as the cause of death. Microscopic lesions are similar to those observed with cardiomyopathy and consist of an increase in fibrous connective tissue around myocardial vessels that extends into the interstitium, atrophy, and loss of myocytes. Secondary hepatic congestion might be present (Campbell and Blair, 1978; Miller and Merton, 1982; Moreland et al., 1986; Parrott et al., 1984).

Dermatomycosis

This is an uncommon disease, and cases occur either in very young animals kept in poor conditions or in older, immunosuppressed animals. Both *Microsporum canis* and *Trichophyton mentagrophytes* have been seen in ferrets.

Grossly, animals have areas of crusting alopecia with brittle hair and numerous broken hair shafts. In immunosuppressed animals, the rash can become generalized.

Microscopically, the skin from affected sites is generally covered with a thick layer of keratin debris, degenerate neutrophils, and entrapped fungal arthrospores and hyphae. There is ulceration of the skin, and follicles often contain numerous fungal arthrospores that occasionally invade the hair shaft. There is generally a neutrophilic or lymphoplasmacytic dermal infiltrate in perivascular and periadnexal areas. *P. carinii* can be induced by cortisone treatment, but natural disease has not been reported (Fox et al., 1998b).

Ectoparasites

Sarcoptic mange comes in two distinct forms in ferrets: a very pruritic whole-body form and a variably pruritic form localized to the feet characterized by swollen feet, evidence of self-mutilation, and nail loss (Phillips et al., 1987). Microscopically, there might be ulceration and hyperkeratosis of the skin and a few cross sections of mites in the epidermis or deep under the overlying crust. Demodectic mange is generally seen in older or immunosuppressed ferrets. Skin scrapings can demonstrate the presence of nymphs or adults, and skin biopsies reveal few cigar-shaped mites within the hair follicles. Ferrets are commonly infected with ear mites (*Otodectes cynotis*) and fleas (*Ctenocephalides* sp.). Most young ferrets and many older ones have clinical cases of ear mite infection that require periodic treatment. Grossly, ferrets with ear mites have copious amounts of a thick brown–black wax, and adult mites and eggs can be found microscopically (Fox, 1988).

Other sporadic bacterial infections reported in ferrets include diarrheal disease due to *Campylobacter coli* (Larson and Hoffman, 1990) and abortion due to *C. jejuni* (Bell and Manning, 1990). *S. aureus*, *Streptococcus pyogenes*, and *Corynebacterium* spp. are infrequent causes of sub-cutaneous and mammary gland abscesses, infected bite wounds, genital infections in females in prolonged estrus, and oral infections secondary to trauma.

Neoplastic Diseases

Ferrets have an incidence and spectrum of neoplastic disease similar to other mammalian species. Endocrine, hemolymphatic, integumentary, and digestive systems were most commonly affected. Tumor incidence is highest in ferrets between 4 and 7 years old. The most common tumor types are pancreatic islet cell, adrenocortical cell tumors, and lymphoma (Li et al., 1998).

Islet Cell Tumors

Pancreatic endocrine tumors are common in the ferret. Clinical signs include lethargy, stupor, ptyalism, and ataxia that can progress to coma and death (Jergens and Shaw, 1989; Lumeij et al., 1987; Marini et al., 1993). Metastasis to visceral organs is rare. Grossly, these tumors are well-defined nodules that range in size from 2 to 1 cm, and they can be multiple. Microscopically, these tumors are unencapsulated and resemble normal, albeit greatly enlarged islets of Langerhans. These neoplasms stain strongly for insulin with scattered glucagon staining (Fix and Harms, 1990).

Adrenal Gland Tumors

Proliferative lesions of the ferret adrenal gland are common in the cortex, and they fall into three categories: hyperplasia, adenoma, and carcinoma. The histological features of these lesions often overlap, and the incidence of nodular hyperplasia is more common than adenoma or carcinoma. The presence of necrosis, cellular atypia, and an increased mitotic rate are strong indicators of malignancy. The presence of a single nodule in the adrenal cortex without factors associated with malignancy indicates adenoma, whereas the presence of multiple nodules is evidence of nodular cortical hyperplasia (Fox and Marini, 1998; Lawrence et al., 1993; Rosenthal, 1993). In addition to cortical tumors, adrenal teratomas are reported in ferrets. The tumors contain tissues from ectodermal, mesodermal, and endodermal germ cell layers including rudimentary teeth (Williams et al., 2001).

Lymphosarcoma

This is the most common malignant tumor in the domestic ferret. It is speculated that lymphosarcoma in the ferret might be the result of a retroviral infection (Erdman et al., 1995). A viral agent has not, as of yet, been isolated from cases of lymphosarcoma in the ferret. Gastric lymphoma resembling gastric mucosa–associated lymphoid tissue (MALT) lymphoma linked with *H. pylori* infection in humans was observed in ferrets infected with *H. mustelae* (Erdman et al., 1997). Different forms of the disease have been recognized. Older ferrets have the well-differentiated lymphocyte form, and young ferrets less than 2 years of age have the large blastic form and a third form that resembles that of the lymphocytic form with a subpopulation of atypical large cleaved, multinucleate lymphocytes and occasional Reed–Sternberg-like cells. Grossly, adults with lymphocytic form have diffuse lymphadenopathy and splenomegaly, and metastatic nodules can be present in a number of visceral organs. The presence of a large thymic mass is strongly suggestive of the juvenile form (Batchelder et al., 1996). Microscopically, in the adult form, there is effacement of the normal nodal architecture by an infiltrate of small noncleaved lymphocytes. In the liver, neoplastic infiltrates are primarily seen extending from portal areas, whereas in the spleen, there is an expansion of the periarteriolar lymphoid sheaths. Nodal involvement is observed late in the disease in the juvenile form, and the population consists of large cleaved and noncleaved lymphoblasts. In the liver, neoplastic cells are more commonly seen as discrete nodules distending sinusoids and replacing hepatocytes, whereas in the spleen, the periarteriolar lymphoid sheath is totally replaced and expanded by a monomorphic lymphoblast population (Erdman et al., 1992).

Chordoma

Chordomas arise in or adjacent to the vertebra from remnants of primitive notochord and are most commonly seen at the tip of the tail and rarely in the cervical spine (Dunn et al., 1991; Williams et al., 1993). Metastasis has not been seen in neoplasms arising in the tail. Grossly, they are seen as club-like swellings at the tip of the tail that involve the last caudal vertebra. Cervical chordomas present as lytic neoplasms in the neck of animals with posterior paresis. Microscopically, the neoplasm is composed of foamy "physaliferous cells" that are separated by a moderate amount of myxomatous matrix with areas of well-differentiated cartilage and bone (Allison and Rakich, 1988; Dunn et al., 1991).

Skin Tumors

The two most common cutaneous neoplasms in ferrets are sebaceous epithelioma and mast cell tumor. Sebaceous epitheliomas appear as warty, verrucous lesions with a predilection for the head and neck, and mast cell tumors appear as pruritic flat, alopecic, hyperkeratotic plaques. Microscopically,

epitheliomas are composed of basal cells, of which a small percentage exhibit sebaceous or squamous differentiation, whereas mast cell tumor is composed of well-differentiated mast cells with low numbers of EOS (Li et al., 1998; Parker and Picut, 1993; Stauber et al., 1990). Multiple progressive piloleiomyomas are not an uncommon disease in older ferrets (Mialot et al., 2010).

Miscellaneous Tumors

Other uncommon neoplasms encountered in ferrets include osteomas of the flat bones, leiomyosarcoma, interstitial cell tumors of the testes, ovarian germ cell or stromal cell tumors, and adenocarcinoma of the pancreas and intestine. Apocrine gland cystadenomas and carcinomas are not uncommon and can occur around the head, neck, prepuce, and vulva, due to the large numbers of scent glands in these regions. Hemangiomas and low-grade hemangiosarcomas are occasionally seen; metastasis has not been reported. Squamous cell carcinoma has been reported several times in the ferret and has a predilection for the face, where it is locally destructive with a low metastatic potential (Li et al., 1998).

Endocrine and Miscellaneous Diseases

Adrenal-Associated Endocrinopathy

This is a common endocrine disorder of middle-aged to older ferrets. The syndrome is the result of proliferative lesions in the adrenal cortex that secrete excess amounts of estrogenic hormones but rarely cortisol. As a result of excess estrogen, affected ferrets exhibit a range of cutaneous, behavioral, and reproductive signs (Fox et al., 1987; Rosenthal, 1993). Grossly, lesions are characterized by bilaterally symmetrical alopecia beginning over the tailhead and progressing forward over the flanks and abdomen. Spayed females can have enlarged vulva. Microscopically, the proliferative lesions of the adrenal cortex can be hyperplasia, adenoma, or carcinoma (Lawrence et al., 1993; Rosenthal, 1993).

Estrus-Associated Aplastic Anemia

Ferrets are induced ovulators, and intact unmated jills will develop marked bone marrow suppression as a result of high levels of circulating estrogens. Initially, there is a mild thrombocytosis and leukocytosis, but the condition soon progresses to a nonregenerative anemia, leukopenia, and thrombocytopenia. The anemia can remain nonregenerative anemia up to 4 months past ovariohysterectomy in affected animals. Hemorrhage is reported to be the most common cause of death. Grossly, female ferrets in estrus have prominently swollen vulvas, and signs of hyperestrogenism include pale mucous membranes, alopecia, melena, thin watery blood, hemorrhages throughout the body, hematuria, pyometra, bronchopneumonia, and vaginitis. Diagnosis of aplastic anemia is most commonly based on the presence of a low PCV (<20%) in an estrus jill. Microscopically, the most characteristic lesion in affected jills is hypocellularity of the bone marrow (Fox et al., 1998b).

Cardiomyopathy

This is a common disease in the American lines of ferrets, which has a presumed genetic basis. Several forms of this condition can be seen: dilatative, hypertrophic, and a restrictive form in which there is marked replacement of myocardium by fibrous connective tissue, with minimal change in chamber area. Signs of cardiomyopathy can be seen as early as 1 year of age in severely affected animals, but are more common between 5 and 7 years of age. Grossly, the heart might appear enlarged, and the right ventricle might appear thin or flabby. With progressively severe cases, there is often an accumulation of a serosanguinous ascitic transudate in the abdominal cavity or the

pleural cavity; congested, occasionally nodular liver as a result of chronic passive congestion; and, in severe cases, atelectatic and compressed lungs due to the presence of a globose heart and abundant pleural effusion. Microscopically, early lesions consist of an increase in fibrous connective tissue around myocardial vessels, which extends into the interstitium. As the condition progresses, there is atrophy and loss of myocytes. Focal areas of myocyte degeneration might be present, with an infiltrate of moderate numbers of inflammatory cells. In some cases of cardiomyopathy, there might be marked focal misalignment of myocytes, suggesting orientation in several different planes (Fox, 1998; Greenlee and Stephens, 1984; Lipman and Fox, 1987).

Gastric Ulcers

Ferrets are extremely susceptible to stress-related gastric ulcers. This is a common finding in animals with other systemic diseases and often contributes to debility in older animals. Gastric ulcers are often seen in association with gastric *H. mustelae* infection; however, a definitive cause-and-effect relationship has not been proven in this species. Grossly, there can be digested blood with multiple pinpoint ulcers or a single large focus usually in the pyloric stomach (Fox, 1998).

Splenomegaly

This is an extremely common finding in middle-aged to older ferrets, and the cause is unknown. Enlarged spleens are prone to rupture. Grossly, there is diffuse enlargement of the spleen, and microscopically, most cases of splenomegaly have marked congestion, florid extramedullary hematopoiesis, and occasionally large areas of coagulative necrosis (Erdman et al., 1998).

Aspiration Pneumonia

This is a common cause of pneumonia in the ferret due to either orally administered medicants or vomitus. Ferrets often resist liquid oral medication by fighting and squirming during administration and often involuntarily inhale part of the medication. Grossly, there might be consolidation of the cranioventral lung lobes, either unilaterally or bilaterally. Microscopically, the lesion is centered around bronchioles, and acute lesions contain neutrophils, sloughed epithelial cells, and bacterial colonies, and in long-standing cases, there might be a pronounced granulomatous response, with multinucleate giant cells. In cases of aspiration of vomitus, the lesion is characterized by extensive necrosis of the airway and surrounding alveoli, with sloughing of the bronchiolar epithelium and coagulative necrosis of the adjacent alveolar septa. Aspiration pneumonia should be differentiated from endogenous lipid pneumonia also known as foam cell foci or subpleural histiocytosis, a common incidental finding at necropsy. This lesion is of no clinical significance and consists of multiple to coalescing white to yellow foci within the subpleural pulmonary parenchyma. Microscopically, the basic lesion is simply an aggregate of lipid-laden macrophages in the alveoli immediately subjacent to the pleura (Fox, 1998).

Chronic Interstitial Nephritis and Urinary Tract Infections

This is a common finding and early lesions can be seen as early as 2 years, and advanced cases resulting in renal failure can occur as early as 4.5 years. Lowering of protein levels after 3 years of age is recommended. Grossly, the kidneys are generally pitted, and large focal depressions can be seen in the outer cortex as a result of scarring. Microscopically, there are linear bands of fibrosis, infiltrates of inflammatory cells, tubular atrophy, and glomerulosclerosis. In addition, bacterial urinary tract infections due to *E. coli* or *S. aureus* are commonly seen in female ferrets. Bladder infections are often subclinical in female ferrets, and ascending infections resulting in pyelonephritis are

not uncommon. Renal failure can result from severe pyelonephritis in this species. Grossly, long-standing cases have hydronephrosis and hydroureter, and microscopically, there is ulcerative cystitis or a suppurative tubulointerstitial nephritis (Fox et al., 1998a).

Diabetes mellitus is a poorly defined, uncommon disease characterized by polydipsia, polyuria, glucosuria, and loss of body condition. Blood glucose levels in affected ferrets generally range into the 500s, but levels as high as 725 g/dL have been reported. Microscopically, glycogenic vacuolation of the islets of Langerhans and glycogen accumulation in renal tubular epithelium could be seen.

Other sporadic conditions have been reported occasionally in ferrets. Neural tube birth defects are reported in kits. The lesions range from simple cranioschisis to spina bifida, to craniorachischisis (Williams et al., 1994). Prostatic squamous metaplasia due to excess estrogens from adrenal lesions has been recognized as a common cause of dysuria and urethral blockage in the ferret. Older ferrets can have broken upper canines, accumulation of dental calculi (moist diet), and tooth root abscess (Andrews and Illman, 1979). Idiopathic megaesophagus occurs in middle-aged to older ferrets with marked dilation of the intrathoracic esophagus, secondary *Candida* infections, and aspiration pneumonia (Blanco et al., 1995). Eosinophilic enteritis is a wasting disease seen in young male ferrets under 14 months of age. The lesion is characterized by eosinophilic infiltrates in the small intestine and mesenteric lymph nodes and rarely with eosinophilic vasculitis (Fox et al., 1992; Palley and Fox, 1992). Young or bored cage-bound ferrets can have GI foreign bodies (Mullen, 1992), renal cysts (Dillberger, 1985), urolithiasis (Nguyen et al., 1979; Palmore and Bartos, 1987), and cataracts (Miller et al., 1993).

METABOLISM

Shayne Cox Gad

The ferret is a relatively new species in toxicological and pharmacological research. There has been increased interest in this species since the early 1970s (Hoar, 1984), more so in Europe than in North America. Interestingly, however, the pace of research specifically on xenobiotic metabolism in this species has declined from a relative flurry of papers published in the late 1970s to relatively few papers published in the late 1980s. As a result, xenobiotic metabolism has not been very well characterized. For example, with regard to disposition, there is no published information on the blood–brain barrier, the blood–testis barrier, or plasma protein binding in the ferret. One has to assume that the ferret is not radically different from other species with regard to these parameters. This review concentrates on the published literature, which tends to fall into three main groups: (1) characterizations of the microsomal mixed function oxidase (MMFO) system, (2) studies to characterize in vivo pharmacokinetics of various chemicals, and (3) studies on species-related differences in conjugation reactions.

Peculiarities of oral absorption in the ferret should be mentioned. The ferret has no cecum, and the transition from the small to large intestine can only be detected by histological examination (Hoar, 1984). The intestine is a relatively short tube, and recent work has failed to isolate any anaerobic GI bacteria. Hence, oral bioavailability of chemicals tends to be less in the ferret than in rodents.

Some indexes of xenobiotic metabolism in the ferret are summarized in Table 7.25. Ioannides et al. (1977) published the first, and one of the few, extensive examinations of the MMFO system. They established that there were definite age-related (day 0–56) increases in, for example, cytochrome P450 and aniline hydroxylase activity. Hence, the data given in Table 7.25 are drawn from adult animals of the age or size that most investigators report using. Ioannides et al. (1977) also reported on slight quantitative strain differences between the polecat ferret and the albino ferret for various parameters. For example, both strains had essentially the same activity toward biphenyl as a substrate, but the albino had much greater activity toward ethylmorphine. Sexual dimorphism was also identified only for the albino ferret, with the males having higher amounts of microsomal

Table 7.25   Summary of Hepatic Xenobiotic Metabolizing Enzymes in Ferrets

Enzyme	Concentration or Activity	Comments and References
Cytochrome P450	0.16–0.73 nmol/mg-m	Ioannides et al. (1977); Lake et al. (1979); Shull et al. (1982); Costello and Chengelis (1989). Strain, sex, and sex variability identified.
Cytochrome b$_5$	0.15–0.22 nmol/mg-m	Ioannides et al. (1977); Shull et al. (1982); Costello and Chengelis (1989).
NADPH/cytochrome C reductase	60–113 nmol/min/mg-m	Ioannides et al. (1977); Lake et al. (1979); Costello and Chengelis (1989).
MMFO activities		
Ethylmorphine demethylase	1.2–3.3 nmol/min/mg-m	Ioannides et al. (1977); Lake et al. (1979); Shull et al. (1982). Good agreement between papers. Costello and Chengelis (1989).
Aniline hydroxylase	0.3–1.3 nmol/min/mg-m	
Benzo(a)pyrene hydroxylase	0.95 ± 0.13 nmol/min/mg-m	
Epoxide hydrolase	No data identified	No applicable publications identified
UDP-glucuronosyl transferase	Limited data available using these substrates	Lake et al. (1979); Shull et al. (1982). Available papers use conflicting data presentations, making comparisons difficult.
1-Naphthol		
4-Methylumbelliferone		
Glutathione S-transferase		
p-Nitrobenzyl chloride	18.2 ± 4.6 nmol/min/mg-c	Costello and Chengelis (1989).
Chlorodinitrobenzene	485 ± 107 nmol/min/mg-c	
Protein content estimates		
Microsomal	10–30 mg/g tissue	Ioannides et al. (1977); Costello and Chengelis (1989); Lake et al. (1977).
Cytosolic	110–150 mg/g tissue	

Note: mg-m, mg microsomal protein; mg-c, mg cytosolic protein.

protein, cytochrome P450, and ethylmorphine N-demethylase activity (Ioannides et al., 1977). In contrast, Shull et al. (1982) reported that although the concentrations of cytochrome P450 were not different, females had higher microsomal activity toward benzo(a)pyrene, hexobarbital, ethoxycoumarin, and ethylmorphine. Given this information, one should assume that there is the potential for strain- and sex-related differences in xenobiotic metabolism in the ferret, and one should consider establishing baseline data for the ferrets being used in specific studies. This is further reinforced by the variability displayed in the sets of clinical chemistry data reported elsewhere in this chapter. P450 levels in the ferret liver are only 30% of those in rat and have been characterized as including 1A1, 1A2, 2D6, 2C19, 2E1, and 3A1 isozymes (Lin and Lu, 1997; Liu et al., 2003).

Depending on the age, sex, and strain of the ferret, relative liver weight ranges from 3.0% to 5.0% of body weight. There is also some variability in microsomal protein from adult animals: Values as high as 33.8 mg/g liver (Lake et al., 1977) and as low as 12.1 mg/g (Ioannides et al., 1977) have been reported. Concentrations of P450 are likewise varied, ranging from 0.19 nmol/mg microsomal protein to 0.73 nmol/mg (values inferred from data presented by Lake et al. [1977] and Ioannides et al. [1977], respectively). However, the values of 0.29 (Lake et al., 1979) and 0.19 nmol/mg microsomal protein (Shull et al., 1982) would appear to be better estimates. Ferrets tend to have less microsomal protein and less cytochrome P450/mg protein than rats. Hence, on a gram liver basis, ferrets have considerably less cytochrome P450 than rats. In general, however, ferret cytochrome P450 has a higher rate of substrate turnover.

The activity on a microsomal protein basis toward common model substrates, such as aniline and ethylmorphine, has been reported to be about the same (Ioannides et al., 1977) or somewhat less (Lake et al. 1979) than the rat. The exception to this observation is that Ioannides et al. reported that the male albino ferret had much higher activities than the rat toward ethylmorphine. This high activity toward ethylmorphine was not confirmed by Lake et al. (1979) or Shull et al. (1982), and the reason for this difference is not clear. These observations again underscore the potential variability in xenobiotic metabolism in the ferret and the importance of establishing baseline values in one's own laboratory.

Only Ioannides et al. (1977) have discussed the developmental aspects of the MMFO. At birth, the hepatic content and concentration of the MMFO are very low. Different enzyme activities develop differently thereafter. Biphenyl 4-hydroxylation parallels the activity of NADPH reductase and reaches adult levels (on a gram liver basis) in 14 days. Other activities tend to follow the development of cytochrome P450, reaching adult levels approximately 2 months after birth. The largest increases in the components of the mixed function oxidase occur after weaning (4–6 weeks of age). Unlike the rat, the mature adult ferret retains measurable biphenyl 2-hydroxylation and p-nitrobenzoate reduction activities.

Few in-depth studies on the inducibility of the ferret MMFO system have been reported. There are no reports on the effects of phenobarbital on xenobiotic metabolism in the ferret. Lake et al. (1979) have demonstrated, however, that Aroclor 1254 (single intraperitoneal dose, 500 mg/kg) will cause large increases in microsomal protein, the content of cytochrome P450, and the specific activity toward aminopyrine, benzphetamine, ethylmorphine, and aniline. In contrast, Shull et al. (1982) have reported that Aroclor 1016 and 1242 (by dietary admixture, 20 ppm, for 28 days) caused increases in relative liver weight without increases in the microsomal concentration of cytochrome P450. In a shorter-term experiment, two doses of Aroclor 1242 (100 mg/kg on day 0, 200 mg/kg intraperitoneally on day 5, and sacrifice on day 10) caused fivefold increases in cytochrome P450, with a shift in the absorbance maximum toward 448 nm. These authors (Shull et al., 1982) concluded that the ferret is weakly inducible. The ferret differs from the rat in that the polychlorinated biphenyl (PCB) induction results in large increases in cytochrome P450 with only modest increases in benzo(a)pyrene hydroxylase.

Lake et al. (1977) reported that a phthalate ester ([di-(2-ethylhexyl)phthalate]) caused increases in liver weight in the ferret, but no increases in microsomal protein. The concentration of cytochrome P450 and MMFO activity actually decreased. Here is an example of how an increase in liver weight does not necessarily mean microsomal induction. It has since been established that phthalate esters induced peroxisome proliferation (Reddy and Lalwani, 1983). Lake et al. (1977) did not examine the more common peroxisomal marker enzymes. Hence, the suitability of the ferret as a model for studying peroxisomal proliferation, or in examining toxicity in the absence thereof, remains to be established.

Very few, if any, papers have been published on the activities of epoxide hydrolase or glutathione S-transferase in the ferret. Traditionally, these enzymes are considered protective in that they react with and deactivate reactive potentially toxic metabolites. Early data discussed by Williams (1972) would suggest that the ferret is capable of forming mercapturic acids in a fashion similar to rodents, at least with chlorobenzene. Preliminary data from our laboratory would suggest that ferret glutathione 5-transferase activity with p-nitrobenzyl chloride and chlorodinitrobenzene (Table 7.25) are less than those seen in rats. An interesting paper by Frederick and Babish (1984) might provide some clues as to the activities of these enzymes relative to those of the rat. These investigators compared the mutagenic activity of uninduced rat and ferret S-9 liver fractions in the Ames assay. Ferret liver fraction had more mutagenic activity (with 2-acetylamidofluorene, cyclophosphamide, and 7,12-dimethylbenzanthracene) than that of the rat. When calculated on a cytochrome P450 basis, ferret S-9 has 5–10 times the activity. The higher mutagenic activity in ferrets could be due to lower detoxification activity, but this remains to be established.

Although glutathione S-transferase reactions have not been extensively studied in the ferret, other conjugative processes have been examined. These include glucuronide, sulfate, glycine, and taurine conjugate formation. In general, as a carnivore, the ferret is more likely to form amino acid conjugates (glycine and taurine), whereas rodents are more likely to form glucuronide and sulfate conjugates. This area was extensively reviewed by Hiram et al. (1977). With regard to the ferret, there has been limited activity in this field since. In general, these investigations were of similar design in that model chemicals, usually an aromatic acetic acid, were administered to a variety of species and the different conjugates identified. For example, Emudianaughe et al. (1978) examined

the metabolism of 2-naphthyl-acetic acid. In the ferret, the main urinary metabolite was the taurine conjugate, whereas this was undetectable in the rat where the glucuronide was the main metabolite. There was also a large species difference in the percentage of dose excreted in the urine, 3% in the rat versus 30% in the ferret. This is not surprising as glucuronides in the rat tend to be actively excreted in the bile. Idle et al. (1978) compared the metabolism of 10 aromatic acids in the rat and ferret. The ferret generated substantial (greater than 5%) amounts of taurine metabolites with seven of the test chemicals, whereas the rat did so only with one (2-naphthylacetic acid). Qualitatively, both species had essentially equivalent ability to elaborate glycine conjugates, although the rat tended to have higher activity for any specific chemical. In contrast, the rat produced little glucuronide with benzoic acid, whereas this was the major pathway for benzoic acid metabolism in the ferret. In their studies on phenoxybenzoic acid, Huckle et al. (1981) reported that the glycine and taurine conjugates were the major metabolites in ferrets, whereas the rat produced no detectable taurine conjugate and the sulfate was the major metabolite. These investigators have further shown that the majority of the glycine conjugative ability lies in the kidney, not the liver (Huckle et al. 1981). Therefore, as a general rule of thumb in working with ferrets, one should expect that amino acid conjugates will be the main metabolites of aromatic acids and that these will be primarily excreted in the urine.

The conclusion of the previous paragraph notwithstanding, the ferret does have the ability to form glucuronides with aromatic alcohols (phenol, naphthols, etc.). Conjugation with this class of substrates simply has not been well studied. Ioannides et al. (1977) examined glucuronide formation using 4-methyl-umbelliferone and detected relatively low activity. Lake et al. (1979) detected much higher activities with 1-naphthol as the substrate. For both these substrates, the reported activity is less than that reported for rats. As in other species, glucuronosyl transferase is largely a hepatic enzyme and is inducible by treatment with Aroclor 1254. Hence, glucuronosyl transferase activity in the ferret has been established, but in-depth study remains to be completed.

Although hepatic metabolism normally accounts for the majority of xenobiotic metabolism, extrahepatic pathways can sometimes be substantially involved in certain pathways. For example, the importance of the kidney in glycine conjugation has been mentioned. Extrahepatic pathways can sometimes be important with regard to toxicological mechanisms. Extrahepatic metabolism has been only marginally explored in the ferret. Lake et al. (1979) reported measurable amounts of benzo(a)pyrene hydroxylase, 7-ethoxycoumarin O-deethylase, and 1-naphthol glucuronyl transferase activities in the intestinal mucosa, kidney, lung, and testes of ferrets. As with the rat, these activities were present at much lower (two to three orders of magnitude) concentrations than in the liver. Extrahepatic activities of benzo(a)pyrene hydroxylase and 7-ethoxycoumarin O-deethylase tended to be similar between the rat and ferret, but rats had 10-fold higher benzo(a)pyrene hydroxylase in the kidney. Rats consistently had higher 1-naphthol glucuronyl transferase activities. Extrahepatic benzo(a)pyrene hydroxylase and 7-ethoxycoumarin O-deethylase activities were induced by Aroclor 1254. Hence, extrahepatic metabolism in the ferret is not dissimilar to that in the rat, and the potential involvement of these enzymes in metabolism and disposition and toxicity should be kept in mind.

The disposition and metabolism of a specific chemical in the ferret has been examined in a few papers. Bleavins et al. (1982) compared the disposition of hexachlorobenzene in pregnant and nonpregnant ferrets. Clearance was greatly accelerated in the pregnant and nursing dams. This was attributed to a high degree of placental and mammary transfer of the chemical to pups. This could suggest that the ferret would be a good nonrodent model for multigenerational studies.

Gorrod and Damani (1980) examined the in vivo N-oxidation of 3-substituted pyridines in various animal species. Interestingly, the ferret more closely resembled the other species examined, with regard to N-oxide formation, than the rat. The ferret excreted approximately 34% of the dose as the N-oxide, whereas this figure was only 10% in the rat. Treatment with 3-methylcholanthrene radically changed this relationship, decreasing the amount of N-oxide to 0.43% in the ferret but

increasing it to 4.2% in the rat. This is an example of not only species differences in metabolism, but species differences in response to hepatic MMFO induction.

Ioannides et al. (1982) examined glyceryl trinitrate (GTN) metabolism in the ferret as part of a broader examination of the effects of species, sex, age, and route of administration on the elimination of this chemical. They observed that there was an excellent correlation between body weight and plasma half-life of GTN (the higher the weight, the higher the half-life). The results from the ferret were in line with this conclusion. Ferrets, however, had lower than expected volumes of distribution, which the authors attributed to the smaller proportion of body fat in the ferret in comparison to other species.

Ideally, one would like to know or be able to compare xenobiotic metabolism in the model species to that in humans. Predictive comparisons between humans and the ferret, however, are particularly difficult because xenobiotic metabolism in the ferret is not as well characterized as that for other species. Very few papers have been published comparing the disposition of specific chemicals in both species. In general, there are few hard rules in anticipating species similarities in metabolism. For example, Williams (1972), in a classic work, noted that the human more closely resembled the rat than the ferret in the metabolism of phenol and benzoic acid. In contrast, the rabbit resembles the ferret in the metabolism of phenol, but the human in the metabolism of benzoic acid. Perhaps, as the MMFO of the ferret becomes better characterized, our ability to predict similarities in xenobiotic metabolism between humans and ferrets will also improve.

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The Dog

Shayne Cox Gad, Mark Morse, and Christopher J. Kemper

CONTENTS

TOXICOLOGY

Shayne Cox Gad

The canine model in biomedical research has served a dual role by helping to provide information on biomedical problems in humans as well as fundamental knowledge of benefit to dogs themselves. Advantages of using the dog in the laboratory were recognized by researchers as early as the seventeenth century. The dog's internal system, organs, and muscles are similar to those of man, a fact that has stimulated the development of canine models in numerous areas such as circulation and cardiovascular research. The relatively large size and longevity of the dog and the high incidence of malignancies inherent in the species make the dog advantageous for sequential studies in individual animals related to the etiology, pathogenesis, and therapy of malignancies (Shifrine and Wilson, 1980). Also, the extensive database available on canine immunohematological parameters has made the dog an extremely valuable animal model for organ transplantation studies, especially in the areas of kidney preservation and the evaluation of immunosuppressant drugs (Shifrine and Wilson, 1980).

History

The history of the domestic dog (*Canis familiaris*) is obscure. While the genus *Canis* also includes wolves, jackals, dingoes, coyotes, and more distant relatives such as various foxes (*Alopex*, *Vulpes*, and *Urocyon*) and the Cape Hunting dog (*Lycaon*), there is no wild species from which the dog is definitely known to derive. The wolf and the jackal have been considered as likely ancestors, although an alternative suggestion has been made that the dog as a species may have arisen as a result of a hybridization between some doglike ancestor and the wolf (Fox, 1965b).

The dog is thought to be the oldest of domesticated animals, a fact which has contributed to the difficulty in attempting to identify its wild ancestors. Skeletal remains place its domestication as far back as 8000 BC (Andersen, 1970). It is likely that for many centuries, the early dog remained of one general type, which was similar to the modern-day Australian dingo (Andersen, 1970). The ability to selectively breed dogs through several generations for specific traits is believed to have been a relatively early "human discovery" and was probably triggered by the recognized benefit of using the dog for "hunting" and scavenging activities. Examples of early breeding efforts include a Pekingese type, which has existed in China for about 4000 years, a mastiff breed depicted in Assyrian sculpture about 600 BC, a greyhound type in Egypt of similar antiquity, and the powerful hunting dogs of early Britain and Gaul.

The division into a multiplicity of breeds is a more recent development of the last 300 years. Great Britain has historically been the country most active in the selective breeding of a great variety of dogs; by the nineteenth century, a full complement of breeds was developed for a variety of tasks, including sporting (setter, pointer, spaniel, and the smaller bull and fox terriers), coursing (greyhound), shepherding (Old English and Scottish Collie), sight and smell hunting (including wolfhound, foxhound, and beagle), and guarding (mastiff) and for use as pets (King Charles spaniel, pug, and Pomeranian).

In scientific research, the use of experimental animals that have an unknown history and are of questionable health is a major concern. From a clinicopathological standpoint, heterogeneous populations of mongrel dogs do not appear to be more variable than homogeneous populations; however, laboratory-bred animals generally show less clinical, gross, and microscopic evidence of disease (Pick and Eubanks, 1965). Because of such concerns, researchers have moved in the direction of using laboratory-bred dogs.

Of the many breeds available, only a limited number possess the qualities (such as moderate size, even temperament, and ease of handling) desired in an experimental dog. Specific breeds that have been used in biomedical research include the greyhound for its well-defined muscles and nerves and large chest cavity, the Dalmatian for its humanlike excretory functions, and the German shepherd for its good bone and joint configuration.

In the general area of toxicological research, the beagle is probably the most frequently used species of dog, although the Pembrokeshire corgi has been advocated as a satisfactory alternative (Noel, 1970). Because of the predominant use of the beagle, much of the following discussion will specifically refer to that species.

Normal Parameters

In toxicological research, determining whether a statistically significant change is biologically relevant can often be difficult. Establishing the existence of an adverse effect is in great part accomplished by making comparisons between treatment and control groups. A great deal of reliance is placed on control data that, in the case of most nonroden toxicology studies, are often collected from a relatively limited number of animals (usually four to six for a 2- or 4-week study). Because of this, it is extremely important to have a well-established database of normal physiological parameters with a good estimate of the expected magnitude of variability.

Growth Curves and Feed Consumption

Table 8.1 and Figure 8.1, respectively, show beagle feed consumption and with weight data over a 1-year period starting at approximately 5 months of age. The biggest relative increase in feed consumption is seen between 4.5 and 6 months of age (Table 8.1). A slower rate of increase is observed over the following 3–4 months, and by about 10 months of age, feed consumption levels off and remains stationary thereafter. This pattern coincides fairly well with the rapid fall in the rate of body weight gain, which begins between 6.5 and 7. 0 months of age (Figure 8.2). By about 12 months of age, animals have almost attained full adult weight. In the beagle, there appears to be an increase in the variability in body weight with increasing age. The average male gains more weight with age than the average female; by puberty, males are about 2 kg heavier than females.

Reproductive and Physiological Parameters

Normal physiological and reproductive parameters are shown in Tables 8.2 and 8.3, respectively. The four phases of the estrus cycle are proestrus, a preparatory phase that leads to estrus; metestrus, which is dominated by the influence of the corpus luteum; and anestrus, a period of sexual

Table 8.1 Feed Consumption in Beagles

Approximate Age Range (Months)	Mean Feed Consumption (95% CL) (kg Dry Diet/Dog/Week)	
	Males[a]	Females[a]
4.5–5.0	2.27	2.05
	(1.6–2.8)	(1.4–2.7)
5.5–6.0	2.62	2.39
	(2.1–2.8)	(1.8–2.8)
6.5–7.0	2.68	2.45
	(2.3–2.8)	(1.9–2.8)
7.5–8.0	2.73	2.55
	(2.4–2.8)	(2.0–2.8)
9.5–10.0	2.76	2.60
	(2.7–2.8)	(2.2–2.8)
10.5–16.0	2.77	2.59
	(2.6–2.8)	(2.1–2.8)

Source: Adapted from Noel, P. R. B., The challenge of selecting the suitable animal species in toxicology, in *The Problems of Species Difference and Statistics in Toxicology*, Vol. XI, Baker, S. B., De, C., Tnpod, J., and Jacob, J., eds., Excerpta Medica Foundation, Amsterdam, the Netherlands, 1970, pp. 57–69.

[a] N = 130.
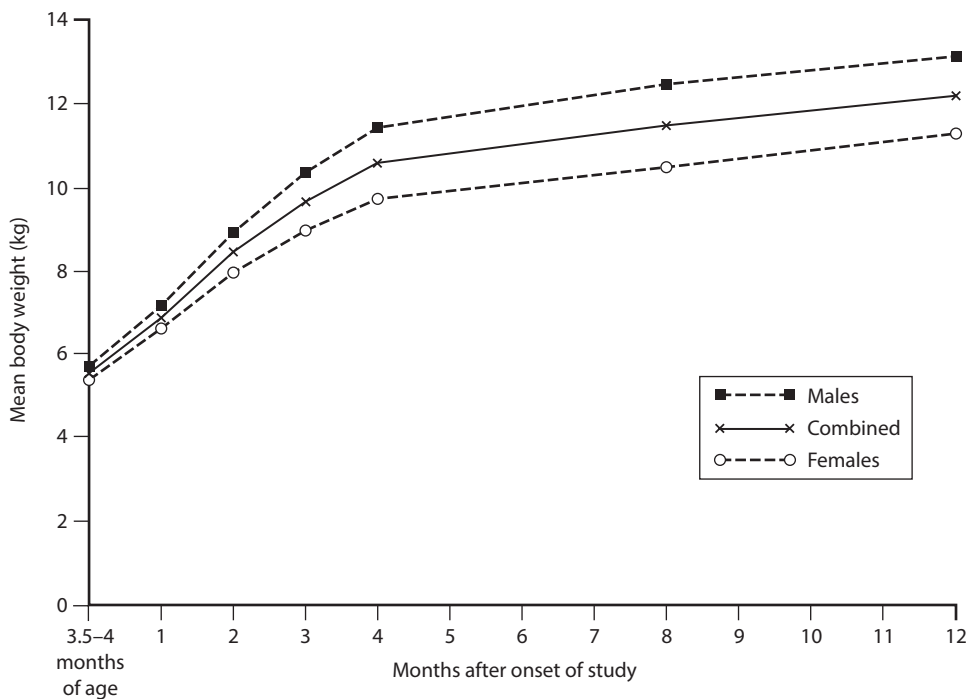
Figure 8.1 Growth weight curves in the beagle. (Adapted from Noel, P. R. B., The challenge of selecting the suitable animal species in toxicology, in *The Problems of Species Difference and Statistics in Toxicology*, Vol. XI, Baker, S. B., De, C., Tnpod, J., and Jacob, J., eds., Excerpta Medica Foundation, Amsterdam, the Netherlands, 1970, pp. 57–69.)

Figure 8.2 Urine collection from dog stainless steel cage.

Table 8.2   Normal Physiological Parameters

Average Life Span	12.5 Years[a]
Daily food consumption	25–40 g/kg body weight
Daily water consumption	Ad libitum; approximately 600 mL
Rectal temperature	37.8°C ± 0.13 (SD)[c]
Respiration rate	20/min (10_30)[b]
Oxygen consumption	0.36 mL O_2/g/h[b]
Tidal volume	24 mL/kg (18–35)[b]
Minute volume	4.5 L ± 0.2[c]
Functional residual capacity	367 mL (248–540)[b]
Lung compliance	117 mL ± 5.6 (5 CM H_2O)
PO_2	73.7 mmHg (61–87)[b]
PCO_2	36 mmHg (29–46)[b]
pH	7.44 (7.37–7.51)[b]
Basal metabolism	2 cal/kg/h[d]

[a] In dogs raised under "protected" environmental conditions (Andersen and Rosenblatt, 1974).
[b] Measured in beagles weighing from 6.8 to 11.5 kg (Pickrell et al., 1971).
[c] Measured in beagles weighing 13 kg (Andersen, 1970).
[d] Michaud and Elvehjem (1944).

Table 8.3   Reproductive Parameters

Puberty	6–12 months of age
Breeding age	
Males	10–12 months
Females	9–12 months
Gestation	60–65 days
Estrus cycle[a]	
Proestrus	5–15 days
Estrus	5–15 days
Metestrus	60–65 days
Anestrus	Length variable
Weaning age	5–8 weeks
Litter size	1–11 pups; 5–6 average
Breeding age (males)	10–12 months
Peak testis development	32 weeks[a]
Peak testis weight	8.6 g[a]
Peak epididymis and deferens gland, penis, and prostate development	32 weeks[a]

[a] Tsutsui et al. (1986).

quiescence (Christie and Bell, 1971). While most mammals can be classified as seasonal or continuous breeders, the dog fits neither category. The observations of cyclical ovarian events and accompanying behavioral and morphological changes in genitalia at one heat period that continue until the next suggest that the bitch has an extended estrous cycle (Engle, 1946; Jochle and Andersen, 1977).

In the male beagle dog, the reproductive organs appear to reach a peak of development at about 32 weeks of age (Tsutsui et al., 1986). This sexual maturation is preceded by the rapid development of the anterior pituitary gland, which in turn stimulates testicular development by gonadotropin release.

Husbandry

Proper handling and care of the experimental dog is essential for its general physical and psychological well-being. While dogs have many of the same general requirements as other experimental animals, special consideration must be given to factors such as space adequacy and degree of socialization.

Housing

Housing is one of the most important features in the physical environment of the laboratory animal and usually consists of a microenvironment, or primary enclosure (the cage or pen), and the macroenvironment, or secondary enclosure (die room containing the cage or pen). The housing system employed should allow adequate space for freedom of movement and postural adjustment and provide a comfortable resting area for the animal. It should also be escape-proof, provide adequate ventilation and access to feed and water, keep the animal dry and clean, and meet biological needs. Ideal housing can be turned into metabolism cages by the addition of trays and movable partitions, thus causing minimal disturbance during feces and urine collection. The most common types of housing for experimental dogs are cages and pens (or runs). The current space recommendations of the National Research Council for laboratory dogs, as published in the 1985 revision of the *Guide for the Care and Use of Laboratory Animals* (NIH, 1996), are shown in Table 8.4.

Table 8.4 Minimum Space Requirements for Laboratory Dogs[a]

Weight	Type of Housing	Floor Area/Animal (cm²)	Height[b] (cm)
<15 kg	Pen/run	0.74	—
15–30 kg	Pen/run	1.12	—
<15 kg	Cage	0.74	81.28
15–30 kg	Cage	1.12	91.44

Source: Adapted from the NIH (National Institutes of Health), Guide for the care and use of laboratory animals, U.S. Department of Health and Human Services, Washington, DC, NIH Publication No. 86-23, 1985.

[a] These recommendations may require modification according to the body conformation of individual animals and breeds.

[b] From the resting floor to the cage top.

The Animal Welfare Act states that primary enclosures for dogs must meet a set of minimum requirements (see 9 CFR Part 6) including a minimum amount of floor space (required floor space in feet calculated as [the length of the dog in inches + 6 in.] × [the length of the dog in inches + 6 in.] divided by 144 and again divided by 144).

Indoor Pens

Indoor pens are excellent for the maintenance of dogs over an extended period of time under controlled conditions. Such kennels are usually constructed with concrete flooring and a drain gutter outside the runs. Typical dimensions for pens that can house up to four dogs are 3′ 8″ wide, 8′ high, and 10′ long. Care needs to be taken to ensure that the surface is not so rough that it abrades the dog's footpads nor so smooth that the floor becomes slippery when wet (due to water or urine) and causes injury to the animals or handlers.

Caging

When purchasing caging, special attention needs to be given to provisions for cage ventilation, drainage, and durability. The most commonly used and recommended materials for dog cages are fiberglass or stainless steel, both of which are durable and easy to clean. Most cages used in research facilities are equipped with automatic watering systems and modified doors with feed bowl holders as well as mobile bases to allow easy movement of the cage racks in and out of animal rooms (Figure 8.2).

In general, the current standard size cages (generally 35″ × 30.5″ × 33.75″ or 35″ × 40.5″ × 33.75″ for larger dogs) used in most research facilities should only be used to maintain dogs for limited periods of time. An alternate to the use of pen/runs are modular and expandable stainless steel dog kennels, which allow the investigator to increase the cage floor space as needed.

Exercise

While the dog can adapt to a wide variety of environments, the question has repeatedly arisen as to whether the degree of physical activity possible in a standard laboratory cage is sufficient for maintenance of normal physiological activity. The Animal Welfare Act mandates that research facilities must develop, document, and follow an appropriate plan to provide dogs with the opportunity to exercise (9 CFR Part 3.8). Additionally, the plan must be approved by the attending veterinarian and must include written standard procedures to be followed in providing the opportunity for exercise. The difficulty arises, however, in establishing a program that will be of most benefit to the dogs.

There are several reports in the literature that indicate there are no differences in clinical laboratory determinations, growth weight, and immunological, electrocardiographic, and ophthalmic parameters for dogs housed continuously in standard-sized cages, large cages, or those released for exercise (Namand et al., 1975; Hite et al., 1977; Campbell et al., 1988). Newton (1972) has also demonstrated that, in terms of the musculoskeletal system, the physiological well-being of cage-confined dogs (relative to dogs housed in pens or those cage confined with access to a pen) is unaffected.

The Animal Welfare Act notes that dogs housed individually in cages, pens, or runs that provide twice the minimum floor space (see section "Housing") or group-housed in cages or runs that provide at least 100% of the required space for each dog if housed separately should provide adequate opportunity for exercise (9 CFR Part 3.8). Analysis of activity patterns of dogs confined in cages of different sizes, however, indicates that cage size has no effect on the amount of time spent sitting, standing, or lying down (Campbell et al., 1988). Regardless of the size of the cage, dogs do not exercise unless humans are in the room. The most beneficial effects are seen when dogs are released and exercised as a group rather than alone. Dogs released alone tend to spend most of their time walking or investigating the area rather than running and jumping. The Animal Welfare Act notes that dogs housed individually in cages where a dog is housed or maintained at a facility without sensory contact with another dog must be provided with positive physical contact with humans on a daily basis (9 CFR Part 3.7).

Socialization

Whatever the kennel design or exercise program used by a research facility, the needs of the confined laboratory dog for a certain level of socialization should be recognized. Both dog–dog and human–dog contact is important. Generally, laboratory dogs are more content if they can share a cage with a companion. One approach adopted by some facilities has been to allow two dogs of the same sex and treatment group daily access to each other's cage from early evening to early morning. This approach, however, is not always logistically possible or scientifically feasible. In situations where experimental design dictates that animals be housed separately, normal behavior can be maintained by permitting visual, auditory, and olfactory access to roommates by appropriate spatial cage arrangement. Early handling of young animals to familiarize them with the laboratory environment is extremely important, since lack of early socialization can result in abnormal behavior later in life (Fox, 1965a). One of the most effective measures to prevent such problems in dogs is frequent human contact, which should continue throughout the animals' adult life.

Sanitation

Good sanitation is essential in an animal facility. The macroenvironment as well as the microenvironment need to kept free of dirt, debris, and contamination. Bacterial, viral, and parasitic problems can be magnified by an improperly maintained facility. Special attention should be given to cracks, rough areas, and depressions, which can harbor moisture and provide shelter for parasite ova and other infectious agents (Bebiak et al., 1987).

Animal rooms, corridors, and storage areas should be routinely cleaned and sanitized. Pens and caging should be mopped or hosed out daily to prevent accumulation of fecal material and general debris. Cages and pens should be scrubbed down with a sanitizing solution about every 2 weeks; cages are rotated and thoroughly sanitized (e.g., soaking the cage in a 5% acid solution for about 20 min and then passing them through a 170°F cage wash). Watering systems also need to be cleaned regularly, either through flushing with water or cleansing with an antibacterial agent.

Another area of special attention is the storage of feed. While most laboratory chow is of good quality and contamination-free when it leaves the manufacturer, biological and chemical spoilage

can result during transit or by improper use. A dry, cool environment is recommended for feed storage to prevent mold formation and insect infestation. Feed should be stored off the floor either in unopened bags or in waterproof, cleanable containers after opening.

Temperature, Relative Humidity, and Ventilation

Special attention must be given to regulation of the macroenvironment as well as the microenvironment in which the laboratory animal resides. Environmental stress in humans and animals has been shown to affect physiological status, which in turn can alter susceptibility to infections and toxic chemicals (Baetjier, 1968; NIH, 1985).

Recommended dry-bulb temperatures and relative humidity ranges for dogs are 64.4°F–84.2°F (16°C–27°C) and 30%–70%, respectively (NIH, 1985). Fluctuations in temperature and humidity can affect factors such as behavior and metabolic rate in dogs (Lusk, 1931). Increases in temperature and high humidity are a particular concern because of the dog's limited capacity to dissipate heat (Norris et al., 1968). Sweat glands in the dog are confined to the foot pads, and the animal primarily responds to high temperatures by panting and, to a lesser extent, by radiation and conduction. It has been shown that a stress zone for dogs, defined as a 1. 1° increase in rectal temperature for all subjects tested, can be created at dry-bulb temperatures greater than 32.6°C (Besch et al., 1984). Exposure to higher temperatures may lead to decreases in feed consumption of normal animals, and it is possible that exposing animals to toxic agents in the presence of elevated temperatures may result in an increase in the incidence or severity of toxicity. Norris et al. (1968) have noted that the dog probably cannot survive for extended periods in time in environments appreciably in excess of 40°C and 40% relative humidity.

Temperature and humidity are also considered to be coincident factors in many diseases caused by chemicals and infectious agents in contact with the skin and can alter cutaneous absorption of compounds, local reaction of the skin to irritants, and reaction to sensitizing agents.

Ventilation control is necessary in order to minimize variations due to climatic conditions, provide adequate oxygen, remove thermal loads, and dilute gaseous and particulate contaminants. The necessary volume of air change per room will depend on its maximum holding capacity (i.e., number of animals in the room and room size). The number of air changes per hour are normally between 10 and 20 (Munkelt, 1948; Runkle, 1964). It has been suggested that recirculated room air can be utilized as long as all airborne contaminants have been removed (NIH, 1985); however, this concept has not proven to be successful, in part because of improper or insufficient maintenance of recirculation systems (Gorton, 1978). Special care should also be paid to the positioning of the room inlet and outlet grills so that air is evenly distributed around the room and to each cage irrespective of its position.

Diet

While the dog is classed as a carnivore, it readily adapts to an omnivorous diet. The digestive tract of the dog is relatively short (compared to other mammalian species), which facilitates the rapid passage of food. Dogs also lack intestinal diverticula, or bacterial harboring sacs, which prevent foods that require microbial breakdown (prior to absorption) from being used effectively. Holding times of food in the beagle stomach, small intestine, and large intestine have been reported to be about 3–5, 1, and 10 hours or more, respectively, coming with a total passage time throughout the gastrointestinal tract of 24 hours (Andersen, 1970).

Dogs require dietary sources of energy, amino acids, glucose precursors, fatty acids, minerals, vitamins, and water. A summary of nutrient requirements for dogs (based on the recommendations of the National Research Council, 1985) is presented in Table 8.5. Dogs do not appear to have a

Table 8.5 Nutrient Requirements for Dogs

Nutrient	Unit	Growth Requirements	Adult Maintenance Requirements
Fat	G	2.7	1
Linoleic acid	mg	540	200
Protein			
Arginine	mg	274	21
Histidine	mg	98	22
Isoleucine	mg	196	48
Leucine	mg	318	84
Lysine	mg	280	50
Methionine cystine	mg	212	30
Phenylalanine–tyrosine	mg	390	86
Threonine	mg	254	44
Tryptophan	mg	82	13
Valine	mg	210	60
Dispensable amino acids	mg	3414	1266
Carbohydrate minerals			
Calcium	mg	320	119
Phosphorus	mg	240	89
Potassium	mg	240	89
Sodium	mg	30	11
Chloride	mg	46	17
Magnesium	mg	22	8.2
Iron	mg	1.74	0.65
Copper	mg	0.16	0.06
Manganese	mg	0.28	0.10
Zinc	mg	1.94	0.72
Iodine	µg	0.032	0.012
Selenium	µg	6	2.2
Vitamins			
A	IU	202	75
D	IU	22	8
E	IU	1.2	0.5
K			
Thiamin	mg	54	20
Riboflavin	mg	100	50
Pantothenic acid	µg	400	200
Niacin	mg	450	225
Pyridoxine	mg	60	22
Folic acid	mg	8	4
Biotin[a]	mg	—	—
B_{12}	mg	1	0.5
Choline	mg	50	25

Source: Adapted from National Research Council, *Nutrient Requirements of Dogs*, Subcommittee on Dog Nutrition, National Academy Press, Washington, DC, 1985.

[a] Dogs have a metabolic requirement, but a dietary requirement was demonstrated when foods from natural ingredients were fed.

specific requirement for carbohydrates; however, in a well-balanced diet, they can utilize large amounts of carbohydrates (up to 70%, dry basis) (Andersen, 1970).

Adult laboratory beagles maintained in a laboratory environment function well with one feeding of standard laboratory chow per day. Dogs tend to bolt their food, and animals on a once-a-day feeding regimen will often eat all their food within a 30–45 min period. However, in order to ensure that the slow eaters have sufficient opportunity to finish most of their food, most laboratories allocate a longer period (2–4 hours) for access to feed.

Although commercially available dog chows provide sufficient amounts of all necessary nutrients, some test compounds may induce deficiencies through loss of appetite, malabsorption, or vomiting that can complicate interpretation of toxic effects. Protein deficiency in the dog results in depressed food intake, growth retardation, hypoproteinemia (associated with edema), and a rough, dull hair coat. Deficiencies in any of the essential amino acids can result in similar clinical signs. Diets low in fat can cause dry, coarse hair and flaky dermatitis (Michaud and Elvehjem, 1944). Inadequate intake or loss of essential minerals (e.g., calcium, phosphorus, potassium, and sodium chloride) can result in problems such as spontaneous fractures, bone loss, osteoporosis, and osteomalacia (calcium and phosphorus); restlessness, muscular paralysis, and lesions of the heart and kidney (potassium); and fatigue exhaustion, inability to maintain water balance, dryness of the skin, and loss of hair (sodium chloride). Vitamin deficiencies, exacerbated by test compound effects, can also induce significant pathology.

Water

The body of dead adult dog contains about 60% water and has a limited capacity to store water (Gaebler and Choitz, 1964). The dog can normally cope with a large fluid intake owing to a readily adjustable urine volume, but the unsalvageable water losses of the body dictate the minimum water intake. Average daily water intake in adult dogs has been determined to be approximately 600 mL (Richter, 1938), and the amount consumed daily has been shown to closely approximate total daily water loss (Gaebler and Choitz, 1964). The individual dog's requirements for water appear to be self-regulated and depends on factors such as the type of feed consumed, ambient temperature, amount of exercise, physiological state, and temperament. For these reasons, in most cases, dogs should be permitted free access to water at all times.

Drinking devices for dogs include pans, water bottles, and, most commonly, automatic watering devices. The water source is usually either tap water or filtered/sterilized water. As with other species, if an automatic watering system is used, the water lines and valves need to be routinely checked to ensure they are working properly and the animal has an uninterrupted supply of water. Whatever the water delivery system used, dogs need to be observed on a daily basis for signs of dehydration so that prompt steps can be taken toward rehydration. Dehydration can usually be detected by checking the elasticity of the skin at the back of the neck. A common rehydration procedure is to subcutaneously administer lactated Ringer's solution (200–400 in., depending on the size of the animal).

Disease

The four diseases of most concern in maintaining a dog colony are canine distemper, infectious canine hepatitis, leptospirosis, and rabies. While various immunization programs can be undertaken, the most common approach is to administer a single multigenic vaccine.

Some of the most common infectious and parasitic diseases observed in dogs are listed in Table 8.6. The most common ectoparasites include fleas, lice, ticks, and mites. Otodectic mites especially can be a problem if neglected; however, treatment can easily be effected by the use of an

Table 8.6   Some Common Parasitic and Infectious Diseases of the Dog

Disease	Pathogenic Agent	Clinical Picture	Common Source of Infection
Coccidiosis	*Isospora*	Primarily a disease of young animals. Diarrhea and dehydration.	Ingestion of sporulated oocysts
Campylobacter enteritis	*Campylobacter fetus* subsp. *jejuni* and *intestinalis*	Watery diarrhea (puppies) and intestinalis.	
Giardia	*Giardia trophozoites*	Disease most important in young dogs. Intermittent or chronic diarrhea, which may persist for several months. Signs associated with malabsorption of nutrients (reduced growth rate, weight loss, dull coat, etc.).	Direct contact with feces
Brucellosis	*Brucella canis*	Females: Abortion in third trimester; generalized lymphadenitis and persistent bacteremia.	Contact with the urine of infected animals
		Males: Orchitis, epididymitis, and prostatis.	
Leptospirosis	*Leptospira interrogans*	Often a sudden onset with anorexia, vomiting, fever, and conjunctivitis followed in a few days by hypothermia, depression, dyspnea, muscle soreness, and oral mucosa hemorrhagic areas. Death (10% of cases) usually related to nephritis.	Direct contact with urine of infected animals
Listeriosis	*Listeria monocytogenes*	Abortion, perinatal infections with CNS signs.	Unknown
Tuberculosis	*Mycobacterium bovis* *M. tuberculosis*	Coughs of long duration; lung and pleural lesions almost exclusively exudative in nature.	Exposure to bovine or human tuberculosis
Dermatomycosis (ringworm)	*Microsporum canis* *M. distortum* (most common agents)	Frequently no clinical signs. If lesions occur, commonly seen as circular alopecic areas surrounded by vesicles, pustules, erythema, or scaling.	Direct contact with the lesions of affected animals
Demodicosis (demodectic: mange, red mange)	*Demodex mites*	Most common in dogs 3–5 months old. Pruritic, alopecia, red lesions around eyes or muzzle. Immune deficiency. Severe infestation can become systemic and prove fatal.	Direct contact with infected animals
Heartworm (canine filariasis)	*Dirofilaria immitis*	Early and moderate heartworm disease: Early tiring on exercise.	
		Advanced disease: Lungs exhibit large emboli, thrombi, and pneumonia, and right heart is enlarged and worm filled. Signs consistent with right heart failure.	Exposure to infected mosquitoes

(Continued)

Table 8.6 (*Continued*)   Some Common Parasitic and Infectious Diseases of the Dog

Disease	Pathogenic Agent	Clinical Picture	Common Source of Infection
Cutaneous larval migrans (creeping eruption)	*Ancylostoma braziliense A. caninum A. duodenaleNecator americanus*	Normally, the parasite is hookworms in dogs. Generally, young dogs show clinical disease. Pale gums, weight loss, failure to grow properly. Tarry feces in heavy infections.	Infection through direct contact with feces of an infected animal or contaminated sand or soil
Dipylidium caninum	*Dipylidium caninum* (common tapeworm of dogs)	Generally asymptomatic. In clinically recognized cases, diarrhea and pruritus around anal area.	Ingestion of infected fleas
Leishmaniases	*Leishmania donovani L. tropica L. braziliensis*	May be harbored in bone marrow, liver, or spleen, resulting in macrophages containing the parasite. In more advanced cases, dermal lesions, emaciation, alopecia, keratitis, and seborrhea. Fatality is common.	Animal bitten by infected sandflies
Salmonellosis	*Salmonella* sp. (many serotypes)	Acute gastroenteritis with diarrhea, vomiting, and resultant dehydration.	Ingestion of contaminated foods or water
North American blastomycosis	*Blastomyces dermatitidis*	A chronic disease in dogs. Depression, fever, anorexia, leading to chronic weight loss, and nonproductive dry coughs. Nodules and abscesses through the lung. Dissemination by hematogenous route leads to destruction of peripheral lymph nodes, bones, and meninges.	Unknown
Rabies	Rabies virus	Two clinical forms in dog: (1) Paralytic form characterized by paralysis of the muscles of the throat and masseter region. (2) Furious form in animals will attack humans, moving or inert objects, or other dogs. Death usually follows within 5 days of these signs.	Contamination of bite wounds by saliva of infected animal in the terminal stages of the disease

Source: Adapted from Bekaert, D. A., *Handbook of Diseases from Dogs and Cats to Man*, California Veterinary Medical Association, Moraga, CA, 1982.

oil containing a nonirritating insecticide. The most serious mange mite is *Demodex folliculorum*, which in some colonies has been detected as a facultative pathogen in about 80% of the animals (Greve and Gaatar, 1964).

Of the endoparasites found in dogs, whipworm is of most concern because of its location in the cecum and colon, which makes treatment difficult.

Coccidiosis (*Isospora* spp.) is a common cause of diarrhea and even death in dogs. Most species of coccidia are self-limiting and nonpathogenic; diffuse hemorrhage of the intestinal mucosa, however, can be seen in dogs infected with *Isospora bigemina* (Andersen, 1970). In dogs, as in many other species, adults are generally more resistant to this disease than young animals.

Giardia species are flagellate protozoa that inhibit the small intestine of a wide range of vertebrates. In dogs (usually young animals), the main sign is intermittent or chronic diarrhea. The disease

can often be difficult to detect because animals may harbor the organism and not shed it in the feces until triggered by a factor such as stress, diet/environmental change, or treatment with a test article.

Heartworm (*Dirofilaria immitis*) can be hazardous in dog colonies because of the toxic side effects associated with administering filaricides. This disease is endemic to the eastern and southern states, and dogs entering a facility should be tested for the presence of microfilaria in the blood. It has been suggested that microfilaria counts above 25/mL of blood warrant euthanasia (Andersen, 1970).

Finally, it should be noted that susceptibility to disease can be enhanced in dogs by factors such as poor sanitation, improper environmental control, number of animals in the primary enclosure and the animal room, and/or inadequate or poor quality diet and insufficient water intake. Personnel who handle dogs must also be trained in proper handling techniques to avoid cage-to-cage or dog-to-handler transmissions of infections.

Quarantine and Veterinary Care

Procedures conducted upon arrival of dogs at a research facility should include the performance of a gross physical examination to look for signs of disease or physical defects, measurement of body weight and rectal temperature, and blood collection for hematological (including microfilaria testing) and clinical chemistry analysis. While colony-bred animals should be fully vaccinated by the breeder, random-source animals will likely need to be vaccinated at the receiving facility. If animals are intended for long-term studies (of more than 1 year duration), they will need to be annually vaccinated for rabies. Most facilities will also prophylactically treat dogs for ear mites. Depending on animal health status, newly acquired dogs are generally maintained in quarantine for at least 2–3 weeks for colony-bred animal and 3–4 weeks for random-source animals. Random-source animals should be well separated from colony-bred animals. All dogs should be regularly examined during the quarantine period and observed frequently for signs of any potential health problems.

Dosing Techniques

It is essential that personnel who are responsible for the dosing of dogs be confident in the handling and manipulation of the animals. The first step is establish a sense of confidence and security between the dog and its handler. During this conditioning period, the handler should crouch down to reduce his or her height and slowly bring his or her hand toward the muzzle so that the dog can sniff it. Next, the hand should move along the neck and hindquarters, and the response of the dog assessed. Most dogs, especially beagles, respond well to such handling.

If possible, it is better during a study to use the same team of people who were involved in the conditioning process. Efforts should be made to distinguish potential "fear biters" prior to selection of dogs for a study. To remove a dog from its cage or pen, the handler should hold the animal by the nape of the neck while keeping the forearm in line with the spine and supporting the animal under the abdomen with the other hand.

Oral

The two oral dosing procedures most commonly used with the dog are gavage and capsule administration. Dietary administration, as commonly used with rodents, is another possible oral route; however, this is not an optimal route of administration in dogs for several reasons. The increased activity of the dog, especially when humans enter or are present in the animal room, can result in a continual problem with feed spillage. Another concern is that dogs are often finicky eaters and sometimes will consume little or nothing during the course of a day while compensating by eating more the next day, which can result in erratic blood levels of test compound.

Capsule

The general approach in the administration of a gelatin capsule to a dog is to first wet the capsule with water, open the animal's mouth, and then place the capsule as far back on the animal's tongue as possible. The muzzle is held closed, the head lifted, and the throat stroked until the animal swallows the capsule. The person dosing the animals should look for evidence of swallowing; almost all dogs will lick their lips after swallowing a capsule. After animals have been dosed, cages should be checked for spit-out capsules and any undamaged capsules readministered. Many facilities include a 1–2-week prestudy conditioning period in which dogs are dosed daily with empty capsules in order to help the animals become familiarized with the dosing procedure. It is not uncommon for dogs treated with certain test compounds to exhibit salivation prior to daily capsule dosing; this is probably a conditioned response in which capsule dosing is related to adverse systemic and/or local effects of the test article.

Gavage

The first step in gavaging a dog is to place a rubber bit between the teeth. An alternate method that can be more comfortable for the dog is to place towels in the animal's mouth. A flexible intubation tube is then inserted into the mouth, back into the esophagus, and down toward the stomach. Correct placement of the tube can be ascertained by inserting the free end of the gavage tube into a beaker of water and checking for the absence of bubbles. Dosing volumes for gavage are generally in the range of 5–10 mL/kg, and fluid should be slowly administered in order to prevent gastric reflux.

Capsule administration is generally the preferred method of oral administration in dog studies, since gavaging is more labor intensive and there is always the chance of gavage error or aspiration. One approach in dealing with compounds that cause extensive vomiting in fasted dogs either by capsule or gavage administration is to allow the dogs access to feed for a few hours prior to dosing. Since the incidence of daily vomiting in control dogs can be as high as 50% (see section "Species Peculiarities"), it is often useful to sham dose animals with a water solution for several days before actually starting a gavage study. This will frequently accommodate animals to dosing and reduce emesis during the study.

Subcutaneous

For subcutaneous dosing, the skin over the side of the neck anterior to the scapula is picked up in a triangular fold and a needle (usually 21-gauge) is inserted into the base of the skin fold parallel to the body wall. The plunger of the syringe is then pulled back gently to ensure that the needle has not gone through both sides of the skin fold or that a blood vessel has not been penetrated. In either case, the needle should be repositioned. The volume administered should generally be in the range of 5–10 mL/kg. In general, the irritation produced by subcutaneous administration is relatively moderate, and the route can be used for limited repeated dose studies. The site of injection can be regularly moved from left to right and front to back in a predetermined pattern of injection (e.g., either side of the neck, either side of the midback, and on either side of the far back close to the rump).

Intramuscular

For intramuscular administration, the injection area (usually the meaty part of the hind leg) is swabbed with alcohol. The injection is made with a 20- or 21-gauge needle, and the maximum volume used should be in the range of 2–5 mL. It is important not to insert the needle too deeply

in order to avoid hitting the sciatic nerve. The plunger of the needle is pulled back to ensure that a blood vessel has not been penetrated; if blood is present, the needle should be repositioned. The site of injection should be rotated during a repeated dose study. An important consideration is the potential for a test compound to induce intramuscular irritation. Because of this, it is advised that muscle irritation studies be conducted prior to initiating a repeated dose study.

Intraperitoneal

For intraperitoneal injection, the dog is usually held in a lateral position, and a small needle (usually a 23-gauge, 3/4 in. needle) is inserted about 1/4 into the lower right quadrant of the belly, taking care to avoid damage to the internal organs. The midline should be avoided, so as to reduce the risk of penetrating the bladder. The needle should be checked for proper placement and repositioned. The volume given should be in the range of 3–5 mL/kg of body weight.

Intravenous

Bolus

The femoral, cephalic, and saphenous veins are commonly used for intravenous administration in the dog. With the dog restrained by a second person, the hair over the vein is shaved (or clipped), swabbed with an alcohol solution, and the vein dilated (by pressure or heat) proximal to the injection site. Usually, a 21- or 22-gauge needle is used, and the maximum volume for an adult dog should be about 3–5 mL/kg. For repeated dose bolus or infusion studies, injections should be kept as low on the vein as possible throughout the study so as to prevent the formation of scar tissue.

The use of a butterfly catheter, to which the syringe is attached, is recommended to help avoid slippage of the needle from the vein in case of movement by the animal. The needle should be inserted in the direction of the blood flow and checked for proper placement by pulling back gently on the plunger of the syringe. The contents of the syringe are slowly injected (over 2–4 min). As with intramuscular administration, the potential for intravenous irritation should be determined prior to starting intravenous toxicity studies.

Infusion

For intravenous infusions lasting up to 2 hours, dogs should be restrained by the use of a device such as a sling (or a stainless steel horse) and a jacket and tether. As with bolus injections, the vein to be infused is dilated by the application of pressure or heat, and an infusion catheter (usually 23-gauge) is inserted into the vein. The needle is withdrawn when blood flows out (thus, ensuring proper placement of the catheter), the catheter and tubing are loosely anchored to the leg with tape, and the catheter is connected to the tubing from the prepared syringe. The infusion pump is preset to the appropriate rate divided by total volume and turned on. During the infusion, the limb being infused should be periodically checked for the presence of swelling or bulge. If this occurs, the infusion should be stopped, the catheter repositioned higher on the vein (or another leg used), and the infusion recommenced. Sufficient slack should be maintained in the catheter so that if an animal moves during the infusion, the catheter will not be pulled out.

For continuous intravenous infusions, a more effective approach may be the use of a vascular access port system although Perkin and Stejskal 1994 report successful use of an externalized medical catheter. A subcutaneously placed rigid multipuncture reservoir with an indwelling catheter is inserted into the jugular vein. The test compound is accessed via catheter tubing encased in a swivel/tether apparatus connected to a jacket harness. The advantage of such a system is that it allows the animal greater freedom of movement during the infusion procedure.

Rectal and Vaginal

Compounds administered rectally to dogs can be delivered in the form of suppositories (emulsion and suspension types), rectal gelatin capsules (solutions and suspensions), or micro-enemas; the most frequently used dosage form, however, is the suspension suppository (De Boer et al., 1982). Microenemas are generally prepared as either a gelatin/saline solution or a simple physiological saline solution and administered in a volume of 0.5–1.0 mL. Suppositories are commonly in the weight range of 0.5–1.0 g, should be short and blunted in shape, and are inserted about 4 cm from the outer rectal sphincter. Liversidge et al. (1986) have also described a device for delivery of suppositories that consists of a stainless steel tube (95 × 2.7 mm) with a plunger to eject the suppository; the use of this device allows the suppository to be maintained in the proper position.

Compounds administered vaginally are usually in the form of suppositories or creams. Creams are usually placed in the vagina by means of an applicator. The vaginal opening is then closed with a plastic alligator clip for a specified period of time. Suppositories and tablets can be inserted vaginally with a plastic tampon–like applicator (Fulper et al., 1987).

Data Collection

Observations and Physical Examinations

Clinical observations and routine physical examinations are integral parts of safety assessment studies. Daily clinical observations in dogs are usually conducted both pretreatment and posttreatment and consist of a home-cage observation to record any signs indicative of poor health status or abnormal behavior (such as motor incoordination/reduced motor activity, tremors, salivation, abnormal feces, etc.). Before making such observations, it is important to be familiar with the spectrum of clinical signs that can be seen in a normal population of dogs. Some examples of clinical signs that are occasionally observed in untreated dogs are shown in Table 8.7.

Physical examinations are conducted less frequently during a study than are clinical observations. The animal is first allowed to move around freely on the floor; and exploratory behavior, motor activity, gait, and general demeanor are evaluated. Next, the animal is moved to a table for examination of the head, including the eyes, ears, nose, mouth, teeth, gums, and tongue (for signs of swellings, abnormal nasal, eye, or ear discharges, tension of facial musculature, salivation, swallowing difficulties, etc.). The animal's body is then palpated for signs of masses and nodal swellings, the urogenital and

Table 8.7   Occasional Clinical Observations in Normal Dogs

Vomitus: Usually clear or brown in color
Soft stool: Loosely formed stool
Mucoid stool: Yellowish or dark in color
Diarrhea: Liquid feces
Nasal discharge: Usually slight runny nose
Injected sclera: Slightly blood shot eyes
Protruding nictitating membrane (cherry eye)
Hair loss: Slight to severe focal or generalized hair loss
Cage sores: Usually between the digits of the paws
Signs associated with females "in heat"
    Vulval bleeding
    Vulval turgidity
    Restless behavior

anal region is examined, and rectal temperature is recorded. Pupillary light, corneal, patellar, wheelbarrowing, and hopping reflexes are usually tested, and righting and auditory responses are recorded.

Cardiovascular Parameters

Blood Pressure

Blood pressure can be determined in dogs by either indirect (noninvasive) methods or by direct determination. An example of a noninvasive method has been described by Weiser et al. (1977). The method requires the placement of an inflatable cuff over the cranial tibial artery and the use of an ultrasonic Doppler sensing device to detect arterial wall motion. Direct determination of blood pressure can be accomplished by cannulation of an artery (such as the femoral artery) with a 22-gauge hypodermic needle that is connected to fluid-filled polyethylene tubing that leads to a pressure transducer (Weiser et al., 1977; Tabaru et al., 1987). Transducer activity can then be monitored on an oscilloscope equipped with a paper recorder.

Blood pressure measurements in unanesthetized dogs can vary widely owing to variables such as exercise, anxiety, and excitement. For this reason, all recordings from conscious dogs should always be performed in a quiet, environmentally controlled room with the animals placed in a comfortable position. Some investigators find it of value to condition the dogs for several days prior to recording blood pressure to lay or sit in a given position. At least three readings are usually made and the average blood pressure determined. Andersen (1970) has reported mean systolic/diastolic values of $98 \pm 28/46 \pm 16$, $121 \pm 40/65 \pm 27$, and $130 \pm 29/67 \pm 8$ for beagles in the age ranges of 2–8, 7–10, and 28–59 months, respectively.

Electrocardiography

Procedure for taking ECGs — While over the last 30 years the use of serial electrocardiograms (ECGs) has become routine in toxicity studies, generally accepted standards for adequate lead systems, recording techniques, frequency of taking samples, and principles for interpretation have not been established (Detweiller, 1980). A 10-lead system, consisting of the bipolar leads (I, II, III), the augmented unipolar leads (aVR, aVL, and aV6), and the unipolar precordial leads (V10, CV6LL (V2), CV6LU (V4), CV5RL (rV2)), has been recommended for dogs (Detweiler et al., 1979). There is some debate as to what is the most suitable body posture for the dog. The right lateral recumbent position (dog restrained on right side with the head and neck held flat and in line with the long axis of the trunk) is the position of choice in many facilities. The sternal recumbent, "sphynx" position and the standing position have been also found to work well. The use of a standardized body position, however, is essential during recording, and the most critical factor is consistent positioning of the forelimbs and scapula in order to prevent alterations in the amplitudes of ECG waves in the various leads and resultant vectors (Detweiler, 1980). Whatever the position used, complete reproducibility in any position is unattainable, probably because of the impossibility of being able to maneuver the heart into the same position each time (Katz et al., 1934; Eckenfels and Trieb, 1979).

For recording purposes, animals should be kept on a warm insulating nonconductive surface, such as a table covered with a rubber mat or blanket. The procedure normally requires two people; one to hold and calm the dog and ensure correct positioning during the recording and one to attach the electrodes and take the ECG. Electrode clips are attached directly to the animal's skin. Stainless steel or copper alligator clips (with the teeth filed down to prevent pinching) are commonly used as electrodes, and the total area of contact should be about 1 cm^2. Suitable electrode contact materials include electrode paste, water, and alcohol. Prior to taking an ECG, the thermal pens for each channel should be checked for correction positioning and to ensure the elimination of extraneous noise

due to improperly attached electrodes. It has been recommended that ECGs be recorded for a total period of 60 s (Detweiler, 1980). For a programmed three-channel 10-lead electrocardiograph, a rhythm strip of any three selected leads should be taken to complete the 1 min of recording.

Many cardiologists prefer to record ECGs at a chart speed of 50 mm/s (rather than 25 mm/s) because the faster speed will "stretch out" the ECG and the time intervals can be measured with greater accuracy (Tilley, 1985). It has been noted, however, by Detweiler (1980) that 25 mm/s is a satisfactory chart speed for dog ECGs, and adequate accuracy can be achieved.

For toxicity studies, ECG records are usually taken by technical personnel on groups of dogs and read at a later time by a cardiologist. The quality of the tracings and decision as to whether to rerun any ECGs will be determined by evaluation of instrument performance, technician error (such as incorrect body positioning or electrode placement), and the presence or absence of artifacts (such as 50–60 Hz interference, muscle tremor, and baseline drift). An acceptable quality tracing should have no errors present, a minimum of two complexes in each lead free of artifact (except minor variations in amplitude), and artifacts present in no more than 115 or 2/10 leads (Detweiler, 1980).

Normal ECG Pattern — Normal values for untreated dogs with aging are shown in Table 8.8. The data show that by about 7–8 months of age, most of the ECG parameters have reached adult values.

Dog ECGs can be highly variable, and it is important to take this factor into account when interpreting ECG findings. Electrocardiographic variations that occur relatively frequently in dogs include changes in direction and amplitude (in the same lead) of P-waves, which appear to be related to nervousness and excitation; changes in the amplitude and reversal of direction of T-waves; and changes in the amplitude or disappearance of Q- and S-waves in all leads (Lalich et al., 1941). Positive T-waves are found more frequently in sitting dogs than those standing or lying down and are more common in lead I and less common in lead U. Variations in the direction of the cardiac vector have been shown even when the same animal position is held correctly (Eckenfels and Trieb, 1979).

Eckenfeld (1980) noted that in serial ECG measurements, individual variability appears to be less than that between (or among) animals.

Heart Rate — The heart rate (number of beats per minute) is usually measured from the R-R interval over a specified period of time. The heart rate has been shown to change during the growing period. At 6 months of age, the heart rate is about 150 beats/min, but it progressively falls over the next 12 months to a value of about 120 beats/min (Noel, 1970). The heart rate is often extremely variable in the dog, and values can range from 45 to 220 beats/min in the conscious mature beagle (Eckenfels and Trieb, 1979). For this reason, ECGs should always be measured in a quiet unstressful setting. There is also the concern that keeping an animal in a fixed position while taking an ECG will cause stress and lead to an increase in the heart rate and an exhibition of other excitement-induced ECG anomalies. The unrestrained standing and the intermediate sitting positions have been found to induce less excitation in the animals than other positions (Eckenfels and Trieb, 1979). It is also recommended that a conditioning period be established prior to study start during which the electrodes are attached and the animals positioned for ECG recording.

Frequent Anomalies in Control Animals — A frequent finding in normal dogs is respiratory-influenced sinus arrhythmia, which is an irregular sinus rhythm that originates in the sinoatrial (SA) node. It is a vagally mediated event and represented by alternating periods of a slower and more rapid heart rate; the heart rate increases with inspiration and decreases with expiration (Eckenfels, 1980; Tilley, 1985). These cyclical changes in the heart rate are often accompanied by cyclic alterations in the amplitude of ECG waves. This type of sinus arrhythmia is well differentiated from nonrespiratory sinus arrhythmia because of the positive correlation with the phases of respiration.

Sinus tachycardia, with a heart rate above 160 beats/min (above 180 in toy breeds and above 220 in puppies), is the most common arrhythmia in dogs. It can most often be explained by nervousness

Table 8.8 Effects of Aging on Electrocardiographic Parameters in Beagle Dogs[a]

		0–2 Months	3–4 Months	4–5 Months	7–8 Months	9–10 Months	11–12 Months	2–3 Years	4–5 Years	6 Years
HR (/min)	Mean	189.1	165.9	146.9	136.5	129.6	130.8	135.8	134.2	134.4
	SD	23.1	24.9	26.0	30.0	28.7	25.7	23.3	22.1	19.3
	Range	153–228	120–202	94–182	80–186	76–178	79–175	76–176	90–174	94–163
	N	131	272	248	118	114	95	56	71	34
PR (ms)	Mean	73.0	83.8	93.7	94.7	98.7	99.7	98.8	104.1	104.4
	SD	8.3	10.4	11.4	12.0	14.4	12.1	11.3	11.4	9.1
	Range	60–84	66–100	74–112	72–114	74–122	78–114	78–116	82–124	82–120
	N	131	271	248	93	114	93	56	71	34
QRS (ms)	Mean	41.8	43.1	44.9	46.7	46.1	47.1	48.4	49.1	50.2
	SD	3.6	4.2	3.9	4.7	3.8	4.7	4.4	3.9	5.0
	Range	36–48	36–48	36–50	36–54	38–50	38–56	40–56	42–56	42–56
	N	130	272	247	117	114	92	56	71	34
QT (ms)	Mean	161.6	156.4	171.1	177.3	181.5	180.1	184.4	184.9	187.6
	SD	13.5	31.2	16.6	15.7	14.9	13.7	13.5	13.6	10.7
	Range	140–190	138–178	150–194	150–202	145–204	154–202	160–202	160–206	158–204
	N	130	270	233	114	109	90	54	68	33
QTc	Mean	285.7	259.3	265.5	263.1	263.3	263.0	273.6	274.0	278.8
	SD	20.0	50.8	26.1	21.5	20.9	23.4	23.4	17.5	18.7
	Range	258–317	228–292	229–293	224–298	222–292	209–296	227–302	244–294	232–308
	N	130	270	233	114	109	90	54	68	33
AXIS (°)	Mean	66.2	74.5	74.3	68.5	70.9	69.3	64.8	68.5	64.7
	SD	36.8	29.3	22.5	27.2	34.0	24.9	32.0	15.8	21.5
	Range	6–105	28–103	32–96	16–92	36–89	33–87	4–88	41–87	3–84
	N	130	271	247	116	113	95	54	71	33

(fear or aggression). Sinus bradycardia is normally vagus dependent and often occurs in calm dogs and those accustomed to the investigator (Eckenfelds and Trieb, 1979).

A wandering pacemaker consists of a shift of the pacemaker from within the SA node or from the SA to the atrioventricular (AV) node (Tilley, 1985). This is a variant of sinus arrhythmia and is a frequent finding in normal dogs. The shifting of the pacemaker within the SA node causes a gradual change in configuration of the P-wave without it becoming negative, whereas shifting of the pacemaker between the SA node and AV junction results in a gradual change in the configuration of the P-wave, which can become positive, biphasic, isoelectric, and negative.

Another ECG finding that can be found in normal healthy dogs is incomplete right bundle branch block (IRBBB) characterized by the presence of a right axis deviation and a large wide S-wave in leads I, II, III, aVF, CV6LL(V2), and CV6LU(V4). It has been found in the beagle as a genetically determined localized variation in right ventricular wall thickness or just as focal hypertrophy of the right ventricle (Tilley, 1985). Studies conducted in this laboratory have indicated the presence of IRBBB in about 20%–25% of a population of young adult beagles in the absence of accompanying cardiac pathology (unpublished data).

Clinical Laboratory Sample Collection

Blood Collection

The most commonly used sites of blood collection in the dog are the cephalic and jugular veins; other veins used less frequently are the femoral, brachial, and saphenous. The advantage of the jugular vein is that it is large and easy to access and therefore suitable for serial sampling in a toxicity study. The collection site is shaved and swabbed with an ethanolic solution, and a 20- or 21-gauge needle is used to collect the sample. The vein is occluded, and the needle is inserted (bevel upward) pointing toward the animal's head; usually, one person restrains the dog and occludes the vein while a second person collects the sample. Once the desired volume of blood has been collected (usually a total of 5–6 mL for clinical laboratory determinations and 1–2 in. at each pharmacokinetic collection time), the needle is withdrawn, and dry gauze applied (with pressure) to the site until the bleeding stops. Because of the potential for commercial diets to affect clinical parameters such as blood urea, glucose, and cholesterol, it is recommended that animals be fasted overnight prior to blood collection (Street et al., 1968).

Urine Collection

In many laboratories, urine collection from dogs is conducted overnight (approximately a 15-hour period) because of the difficulty in consistently obtaining sufficient volumes of urine in some dogs during shorter collection periods. There are problems, however, associated with such a protracted collection time. Urine casts can dissolve, bacterial activity increase, and bilirubin break down to biliverdin with exposure of the sample to light. Since only a small sample is needed for urinalysis, it is recommended that one be collected from each dog as soon as possible during the collection period. Collection containers should also be protected from light. Common urine collection systems for dogs are metabolism cages or stainless steel troughs that attach to the home cage. When collecting samples, care should be taken to avoid males being housed in cages directly above those of females because of the possibility of the males urinating along the side of the cage and contaminating the sample being collected in the lower cages.

Ad libitum access to water can also be a problem because of accidental dilution of the urine sample. Consequently, it is common for the dogs not to have access to water during the overnight collection period. It is recommended, however, that dogs have free access to water on the following day prior to dosing. Work done in this laboratory to examine the effects of water rehydration on erythrocyte parameters has shown that 30–60 min is a sufficient rehydration period (Guy, 1989).

Common Study Protocols and Associated Considerations

Because of the large amount of background data available on the dog, in particular the beagle, it is a commonly used nonrodent species in acute, limited repeated dose (2- or 4-week studies), subchronic (up to 13 weeks of duration), or chronic (26 weeks or longer) toxicology studies. Examples of experimental designs and suggested timing of various study activities for 4- and 13-week toxicity studies are shown in Tables 8.9 and 8.10, respectively.

For teratology studies, the dog does not appear to be as sensitive an indicator of teratogens as other nonrodent animal models such as primates (Earl et al., 1973) and ferrets (Gulamhusein et al., 1980). Likewise, for studies aimed at evaluating reproductive function, the dog is not the species of choice, primarily because fertility testing is virtually impossible to conduct owing to prolonged anestrus and the inability to predict the onset of proestrus. Also, there is no reliable procedure for

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Table 8.9   Four-Week Toxicity Study

Experimental design

    4/sex/group; 4 dosage groups + I control

    Dose daily for 29 or 30 days

    Sacrifice days 29, 30, or 31

Study activities

    Daily observations: Pretreatment and twice daily during the study period

    Physical examination: Pretreatment and after dosing during weeks 2 and 4

    ECG: Pretreatment and after dosing during weeks 2 and 4

    Ophthalmic examination: Pretreatment and during week 4

    Body weight: Pretreatment and weekly, for moribund animals, and the day of scheduled sacrifice

    Feed consumption: Pretreatment and weekly

    Clinical lab: Twice before the first dosing day, before dosing on day 2, during week 2, and prior to sacrifice

    Urine collection: Pretreatment and during weeks 2 and 4

    Pharmacokinetic sample: Blood collected at specified times after dosing on days 1 and 28

Table 8.10   13-Week Dog Toxicity Study

Experimental design

    8/sex/group; 3 dosage groups + control

    Dose daily for 91, 92, or 93 days

    Sacrifice main group (6/sex/group), week 14

    Sacrifice reversal group (2/sex/group), week 18

Study activities

    Daily observations: Pretreatment, twice daily during treatment phase, and daily during reversal

    Physical examination: Pretreatment, after dosing during weeks 4, 8, and 13 of treatment, and during week 4 of reversal

    ECG: Pretreatment, after dosing during weeks 4, 8, and 13 of treatment, and during week 4 of reversal

    Ophthalmic examination: Pretreatment, during weeks 6 and 13 of treatment, and during week 4 of reversal

    Body weight: Pretreatment (3 times), weekly during the treatment and reversal periods, for moribund animals, and at scheduled sacrifice

    Feed consumption: Pretreatment, weekly through first month, bimonthly during the remainder of the treatment period, and weekly during reversal

    Clinical lab: Pretreatment, during weeks 4 and 8 of treatment, day 1 of reversal, for moribund animals, and prior to scheduled sacrifice

    Urine collection: Pretreatment, monthly during treatment, and during week 4 of reversal

    Pharmacokinetic samples: Blood collected at specified times after dosing on days 1 and during weeks 6 and 12

the induction of estrus or ovulation. Although semen is relatively easy to collect from the dog, the number of sperm in an ejaculate can vary widely owing to factors such as age, testicular size, and season (Amann, 1982).

The dog, however, is an appropriate nonrodent animal model for use in pediatric studies to assess the effects of various treatments on postnatal development (equivalents of human neonate, infant, child, and adolescent developmental phases).

Selection of Study Animals

Age

It has been recommended that, depending on the age of the human population projected to be exposed to a given compound, dogs should be at least 4–6 months and no more than 9 months of age at study start (Mosberg and Hayes, 1989).

For pediatric studies, judicious selection of even younger animals for standard repeated dose toxicology studies may obviate the need for a special pediatric study in the dog. In many laboratories, dogs are usually in the age range of 7–9 months at study start; however, for short-term studies (acute 2 or 4 weeks), there is usually no problem with using older animals (up to 12 months of age).

Pretreatment Evaluation

It is imperative that dogs be properly screened prior to assignment to a study. Animals should be selected on the basis of acceptable findings from body weight, physical, ophthalmic, and electrocardiographic examinations as well as urinalysis, clinical chemistry, and hematological evaluations. Pretreatment evaluation should take place within 3–4 weeks of study start, and it is recommended that clinical laboratory determinations be made close to the start of the study. Additionally, it is advisable to limit the number of siblings that are assigned to a study to no more than one per sex per treatment group.

Numbers of Animals and Experimental Design Considerations

The number of animals assigned to a treatment group will depend on the duration of the study (see Tables 8.9 and 8.10). In general, fewer animals are used for a 2- or 4-week study (suggested "N" is 4 animals per sex per group; no reversal group) than for a 26-week or 1-year study (suggested "N" is nine animals per sex per group; includes two to three dogs per sex per group for reversal). Whichever type of study is conducted, the number of animals should be equal across sexes for any given treatment group.

Often, there is little information on repeated dose toxicity of a test compound at the time 2- or 4-week dog toxicity studies are conducted, yet findings from these studies are often the main basis for dose selection for the more long-term studies. Thus, it is recommended that four (rather than three) dosage groups be used in these studies in order to obtain a more complete toxicity profile of the compound.

Randomization

Dogs are usually randomly assigned to treatment groups using a blocking procedure with blocks defined from stratified body weights. Because of possible inherited susceptibility or resistance to toxic effects induced by a test compound, the distribution of siblings needs to taken into account when reviewing the final randomization. A general rule is to limit the number of siblings to one per sex per group. This can be achieved by placing a limit on the number of siblings (i.e., not to exceed

the number of groups in the study) that will be accepted from the animal supplier. Random assignment of reversal animals is usually done prior to study start; however, if any adverse findings are detected during the treatment phase, the study director may want to include some of the affected animals in the reversal group.

Study Activities

When scheduling activities for dog studies, it should always be taken into consideration that most, if not all, study-related activities will be conducted in all animals. The repeated manipulation of dogs for blood collection as well as for ECG, ophthalmic, and physical examinations will likely induce some stress in the animals. For shorter-term studies, this can be a concern, especially at the beginning and end of a study where there are multiple study activities to be conducted in a relatively short period of time (see Table 8.9). Efforts should be made to separate the various study activities so that no more than two activities are scheduled on the same day.

Species Peculiarities

Emesis

Dogs have a natural tendency to vomit. While this may be easier to explain in the pet dog that has a greater chance of ingesting foreign materials and spoiled foods, emesis is often seen in the laboratory dog living in a controlled environment. For a given study, the incidence of vomitus in control animals on a single day can be as high as 40%–50% (unpublished data). The cause of this emesis is unclear, but it is probably related to the inherent excitability of the dog. Vomiting can be a particular problem when a drug is given orally, since a portion of the dose may be lost.

Because vomiting during a study can contribute to the variability of the pharmacokinetic results for that study, it is recommended that all occurrences of vomiting be recorded and additional observations be made on days when pharmacokinetic blood sampling occurs.

Shedding and Alopecia

Shedding is frequently seen in beagles. This is in part seasonal in that it occurs in both sexes primarily in the spring and, in females, is in part estrous related (Al-Bagdadi et al., 1977). A more serious problem arises when dogs develop pruritic alopecia, which occurs at a greater frequency in the summer and appears to be more common in females. Clinical signs include bilateral alopecia of increasing severity, pruritic ears progressing to thickening of the skin around the ears, face, and neck, and skin inflammation in severe cases.

Polysorbate-Induced Histamine Reaction

Intravenous administration of polysorbates (Tweens) 20, 40, 60, and 80 in dogs have been found to cause a release of histamine or a histamine-like substance that results in a profound cutaneous response (includes reddening of the muzzle, inside of the ears, and sometimes of the general body as well as swollen and bloodshot eyes), scratching, vomiting, signs of weakness and postural difficulties, and hypotension (Krantz et al., 1948). These effects have been seen in this laboratory after administration of a 0.1% aqueous polysorbate 80 solution at a volume of 5 mL/kg (unpublished data). Polysorbate 80 has also been demonstrated to be a potent cardiac depressant in dogs. This allergic depressor response is not seen in the guinea pig, cat, rat, rabbit, or humans (Krantz et al., 1948).

Table 8.11   Use of the Beagle in Safety Assessment Studies

Advantages
    Medium size
    Moderate length of hair coat
    Even temperament and friendly disposition (easy to handle)
    Adaptability to living in groups
    Easy to work with (e.g., dosing, blood collection, ECG)
    Satellite animals not needed for serial blood collection
Disadvantages
    Variation in size and body weight
    Loud, penetrating bark
    Cost of acquisition and maintenance
    Greater test compound requirements than smaller species
    Availability
    Exercise and housing requirements

Advantages and Disadvantages of the Dog

There are well-defined advantages and disadvantages in working with the beagle (Table 8.11). The suitability of the beagle as an experimental model arises from factors such as its medium size and even temperament. The relative ease of handling the beagle makes it possible to be able to perform any routine study-related activities such as serial collection of blood and electrocardiographic examination in the conscious animal.

Disadvantages in the use of the beagle include an often wide variation in size and body weight and a loud penetrating bark, which can be controlled by partial ventriculocordectomy, a procedure that usually gives permanent results but does not affect the well-being of the dog (Andersen, 1970). However, ventriculocardectomy is not a common practice in research facilities. Housing and exercise requirements for the laboratory dog can be a disadvantage in terms of the large amount of laboratory space and additional personnel needed to maintain and exercise animals on study. This is particularly relevant in light of revisions in the Animal Welfare Laws of many countries (e.g., U.S. Department of Agriculture), which have resulted in increased space requirements for dogs and/or a specified periods of daily exercise. Additionally, test compound requirements are higher for the dog (relative to the rat, guinea pig, ferret, and monkey), which can often be a major disadvantage when only limited quantities of test compound are available. As mentioned in the section on species peculiarities, the high incidence of vomiting and the histamine release reaction to intravenous injection of Tweens can also be disadvantages. Finally, it should be recognized that experiments using large numbers of beagles usually cannot be set up quickly, and careful advance planning is needed to ensure that animals of the right age range and number can be obtained and housed in time to meet projected deadlines.

PATHOLOGY

Mark Morse

This section on the pathology of the laboratory dog is not intended to be a comprehensive treatise on canine pathology, but primarily as a source of information on spontaneous gross and microscopic changes seen in beagle dogs. Emphasis is placed on the young adult beagle because they are most frequently used as a nonrodent species during short-term safety studies; although, they are also well suited for selected longer-term research studies. Commercial breeding colonies provide

standardized beagles for research and safety studies. Hopefully, the information in this chapter will be useful not only to study pathologists but to those who assist them in the postmortem laboratory and also to toxicologists and students of pathology and toxicology. The material is presented on an organ system basis, with a review of gross and microscopic anatomy, suggested necropsy procedures, a summary of reported spontaneous pathology augmented by the writers' own experience, and selected examples of induced or spontaneous changes useful as models of human disease. For a tabular summary with available incidence rates for gross and histopathological findings in control laboratory dogs, refer to Peckham (2002). Disease problems in laboratory dogs from random sources and conditioned for research studies were reported by Ringler and Peter (1984) and Pick and Eubanks (1965). Also, spontaneous diseases and findings in the laboratory beagle have been reported by Fritz et al. (1966, 1967), Andersen (1970), Hottendorf and Hirth (1974), Maita et al. (1977), Oghiso et al. (1982), and Glaister (1986). The pathobiology of the aging dog is described in detail in two volumes edited by Mohr et al. (2001). Additional information and more detailed descriptions of canine anatomy, physiology, parasitology, infectious and noninfectious diseases, and pathology are available in a number of veterinary medical textbooks (see Aiello [1998], Bonagura [2000], Bonagura and Kirk [1995], Carter [1993], Ettinger and Feldman [1995], Jones et al. [1993], Jubb et al. [1992], Summer et al. [1995], and Urquhart [1996]).

Integumentary System

Anatomy and Histology

The entire body of the dog is covered by an organ known as the integument, or skin (integumentary system), which includes epidermis, dermis, hair, hair follicles, sweat and sebaceous glands, digital pads, claws, and mammary glands (Calhoun and Stinson, 1976). The integument is bound to underlying fascia and skeletal muscles by a subcutaneous, or hypodermal, layer (subcutis, hypodermis) of loose and adipose connective tissue that is not part of the integument but which is often removed with it during dissection. The integument is continuous at all natural body openings with the mucous membranes of the digestive, respiratory, and urogenital systems. At the margins of the eyelids, the stratified squamous ectoderm of the skin becomes the mucous membrane (conjunctiva) of the eye. The outer layer of the tympanic membrane (stratum cutaneum) is very thin without hair or glands (Breazile, 1976). The integument is a large organ. In the newborn puppy, the skin, hair, and subcutis represent 24% of the total body weight (Lovell and Getty, 1967). At maturity, the same tissues represent 12% of the body weight. The principal function of the skin is to separate and protect the body from the environment. Additional functions include temperature regulation, sensory perception, blood pressure control, secretion, storage, and synthesis of vitamin D (Muller et al., 1983). Although the functions of the integument are similar in various laboratory animals, significant morphological differences exist between animals and significant regional differences exist within an animal. Adam et al. (1970) have illustrated many of the regional differences in the beagle. Some regional differences are subtle and are best recognized utilizing carefully collected age, sex, and site-matched specimens.

Epidermis, Dermis, and Hair Coat

The thickness of the skin (epidermis and dermis) and density of the hair coat (pelage) vary in a consistent way over the body. Both skin and pelage are thickest over the dorsal and lateral surfaces of the trunk and lateral surfaces of the limbs and are thinnest on the ventral surface of the trunk and medial surfaces of the limbs (Calhoun and Stinson, 1976). As a separate layer, the epidermis is generally the thinnest in well-haired areas and the thickest in hairless areas. In contrast, the dermis is

usually thickest in well-haired areas and thinnest in hairless areas. In the beagle, the thinnest epidermis is found in the cheek and thinnest dermis is found in the scrotum (Warner and McFarland, 1970).

The hair coat of the beagle is typically tricolored, consisting of black, white, and shades of brown (Warner and McFarland, 1970). Individual variation is great and some beagles are bicolored. Beagles tend to shed hair throughout the year, although some have seasonal molts. Dogs have three general types of hair: tactile, coarse, and fine. Tactile hairs include sinus hairs (vibrissae, whiskers) and tylotrich hairs. Sinus hairs function as slow-adapting mechanoreceptors and are located on the muzzle, upper and lower lips, chin, intermandibular space, near the oral commissures, and above the eyes (Warner and McFarland, 1970). Eyelashes (cilia) are large hairs but are not sinus hairs. Sinus hairs have large follicles containing an endothelial lined blood sinus between the two sheaths of the follicle (Calhoun and Stinson, 1976). Tylotrich hairs are stout hairs scattered among ordinary hairs and function as rapid responding mechanoreceptors (Yager and Scott, 1985). Tylotrich follicles are large, contain a single tylotrich hair, and have a ring of neurovascular tissue at the level of the sebaceous gland. The tylotrich follicle is associated with a focal area of epidermal thickening (tylotrich pad) resting upon a layer of well-vascularized and innervated connective tissue (Yager and Scott, 1985). Ordinary coarse (primary, guard) and fine (secondary) hairs comprise the bulk of the pelage and grow in compound follicles in the dog with a single long coarse hair and several fine hairs emerging from a single opening in the skin. As many as 15 hairs may grow in one follicle (Calhoun and Stinson, 1976).

Sebaceous and apocrine sweat glands are the two principal skin glands in the dog (and mammary gland in the female).

Sebaceous sweat glands. Sebaceous glands are simple alveolar glands whose oily secretion (sebum) results from complete disruption of cells (holocrine secretion). Sebaceous glands are located in the superficial dermis where each gland empties into the upper part of a hair follicle or onto the surface of the skin in hairless areas. Each primary hair has its own sebaceous gland. Secondary hairs usually share sebaceous glands. A ring of sebaceous glands opens into the follicle of tactile hairs (Muller et al., 1983). Where hair is dense, sebaceous glands tend to be long and narrow. Sebaceous glands are larger where hair is sparse and are the largest where associated with small hairs and at mucocutaneous junctions such as the lips and prepuce.

Special sebaceous glands. The dog has several special sebaceous glands. Two types are found in the eyelids. Meibomian glands lie within the tarsal plates of the upper and lower lids. Secretion from Meibomian glands seals the lid margins when the eyelids are closed and prevents overflow of tears when the eyelids are open (Kuwabara and Cogan, 1977). Sebaceous glands associated with the eyelashes are known as the glands of Zeis.

A complex arrangement of glands is found in the anal region of the dog.

Circumanal glands. The largest and most widely distributed glands in the subcutaneous perianal region are known as circumanal, or perianal, glands. Circumanal glands are bipartite glands with a typical sebaceous gland superficial portion and a larger, deeper nonsebaceous portion. The sebaceous portion is functional and empties into hair follicles through patent ducts. The nonsebaceous portion is apparently nonfunctional and is connected to the sebaceous portion by nonpatent "ducts" (Calhoun and Stinson, 1976). The nonfunctional portion comprises large cells that resemble hepatocytes, causing them to be referred to as hepatoid cells (Yager and Scott, 1985). Circumanal glands have been found ectopically in a number of sites, including the tail, flank, back, prepuce, and chin (Yager and Scott, 1985) and walls of anal sac ducts (Miller et al., 1967). Circumanal glands continue to grow throughout the life of the noncastrated male.

Tail gland. Large, well-developed sebaceous glands are also found in a circumscribed oval area of the skin on the dorsum of the tail, a short distance from its base. This gland complex is known as the tail, or supracaudal, gland (Calhoun and Stinson, 1976). Large apocrine glands are also present in this area. The tail gland is marked grossly by stiff hairs and a yellow, waxy appearance to the skin surface (Lovell and Getty, 1967).

Apocrine Sweat Glands

Apocrine sweat glands, as with sebaceous glands, are located throughout the skin of the dog, mainly in connection with hair follicles. The bodies of apocrine sweat glands are located deeper in the dermis than sebaceous glands; however, their ducts enter follicles above the ducts of sebaceous glands. Only one sweat gland is associated with each hair follicle complex. Apocrine sweat gland secretions are scant and rarely perceptible in the dog (Calhoun and Stinson, 1976). Specialized apocrine sweat glands are found in the eyelids, external ear canal, and anal region. In the eyelid, rudimentary sweat glands are associated with the eyelashes and are known as the glands of Moll (Getty, 1967).

Ceruminous Glands

The apocrine sweat glands of the external ear canal are known as ceruminous glands but are apparently only partly responsible for the waxy secretion known as cerumen (earwax). Cerumen appears to be a product of sebaceous and apocrine sweat glands with desquamated epithelial cells as an additional ingredient (Warner and McFarland, 1970). Large apocrine sweat glands are present within the walls of the anal sacs. The ducts of anal sacs also contain sebaceous glands making the anal sac contents a mixture of sebaceous and apocrine gland secretions. Apocrine sweat glands, known as anal glands, also open directly into the intermediate zone of the anal mucosa (Warner and McFarland, 1970).

Eccrine Sweat Glands

Eccrine (merocrine) sweat glands are limited to the footpads of dogs. They have no thermoregulatory function (Muller et al., 1983).

Mammary Glands

Mammary glands are compound tubuloalveolar glands located in the ventral thoracic, abdominal, and inguinal skin of female dogs. The secretory product, milk, is produced by both apocrine and merocrine secretion (Calhoun and Stinson, 1976). The bitch typically has 10 mammary glands arranged in two symmetrical rows. Teats indicate the location of the glands in the male and immature female. One or more glands may be missing. Nine is the usual number of functional glands in the beagle (Sekhri and Faulkin, 1970). Supernumerary teats are common. Mammary glands usually show a size gradient from small (thin) anterior glands to large (thick) posterior ones. The cranial two pairs of glands are the cranial and caudal thoracic glands or simply, glands 1 and 2. The next two pairs are the cranial and caudal abdominal glands, or glands 3 and 4. The most caudal pair are the inguinal, or pubic, glands, or glands 5. The mammary glands are under hormonal control, which regulates development at puberty, sequential changes during the estrous cycle, and growth during pregnancy. Not all parts of active glands are necessarily in synchrony and there may be substantial variation in the size of ducts and alveoli, luminal content, and the character of lining cells. Detailed descriptions of the mammary glands during all stages of the estrous cycle have been published (Sekhri and Faulkin, 1970; Nelson and Kelly, 1974). The selection of mammary gland specimens for microscopic examination should be consistent not only for the purpose of group comparisons but also for correlation of mammary gland and reproductive tract histology. Nelson and Kelly based their observations on specimens from the inguinal glands. Sixty to sixty-five percent of mammary tumors are found in the caudal abdominal and inguinal mammary glands (Crow, 1980; Moulton, 1990), a fact to consider in the design of long-term studies.

Necropsy and Laboratory Techniques

In anesthetized animals, a general examination of the skin should be done prior to exsanguination. All of the body surface, including the mammae, should be palpated to locate masses and to evaluate the texture, elasticity, and thickness of the skin. The luster and texture of the hair should be noted. Abnormal coloration (pallor, erythema, jaundice) should be looked for in lightly pigmented and sparsely haired regions such as the axillae and inguinal region. The perineal region should be examined as well as the surfaces of the pinnae, visible portions of the ear canal, interdigital spaces, and footpads. If no significant abnormality is revealed by the general examination, a single specimen of inguinal or posterior abdominal skin that includes the mammary gland may be collected as a representative sample of the epidermis, dermis, and glandular anexa. If specimens of diseased skin are taken, adequate margins of normal skin should be included. Generally, hair should be removed from skin samples to facilitate processing and sectioning. Hair is preferably removed prior to exsanguination. Whether hair is removed by clippers or scissors, the work should be done carefully so as not to disturb or remove surface pathology or create artifacts. Skin specimens should be flattened prior to fixation. This may be accomplished by carefully stapling the specimen, with the subcutaneous surface down, to small pieces of cardboard or corkboard. The boards may be labeled to identify multiple specimens. Fixation in a flattened position allows proper anatomical orientation and prevents artifacts associated with curling and folding. Three to four millimeter wide blocks of tissue may be excised from the fixed tissue for embedding, leaving a largely intact and labeled specimen for archival storage. Ten percent buffered formalin is the preferred fixative for skin specimens (Muller et al., 1983; Hargis, 1988). Hematoxylin and eosin (H&E) is the most widely used stain, but acid orcein–Giemsa is also recommended as a routine stain for skin biopsies (Muller et al., 1983). The latter authors list several special stains that are useful in dermatopathology.

Special investigations, such as those utilizing the bitch as a test animal in which to study the effects of contraceptives on mammary tissue, may require a detailed examination of the mammary glands. An approach reported by El Etreby and Wrobel (1978) utilized the right mammary system as a whole mount for gross examination and the left mammary system for microscopic examination. Utilizing a modified technique of Cameron and Faulkin (1971), the entire right mammary system was fixed in Tellyesniczky's solution. After removal of the skin and additional fixation, the mammary complex was defatted in acetone, stained with hematoxylin, and cleared with methyl salicylate. The whole mount was weighed and examined by dissecting microscope and photographed utilizing transmitted light. Specimens of the left mammary system were fixed in Carnoy's, Bouin's, or paraformaldehyde solutions and stained by a variety of stains for histomorphology and histochemistry. Frozen sections were also utilized to demonstrate lipids and enzymes. Other approaches to examining the entire mammary system include slicing formalin fixed glands at 5 mm intervals and examining each slice for gross abnormalities (Nelson et al., 1973) or obtaining specimens from palpable nodules in addition to routine sampling of normal glandular tissue (Giles et al., 1978). Microscopic examination of lymph nodes draining the mammary glands is rarely indicated in short-term, routine studies. Nevertheless, knowledge of the usual lymphatic drainage pattern is desirable. The following information was obtained from Christensen (1967) and Moulton (1990). Mammary glands 1 through 3 drain to the axillary lymph nodes on the same side. Glands 4 and 5 drain to the superficial inguinal nodes on the same side. The lymphatics of glands 3 and 4 sometimes connect, allowing both anterior and posterior flow of lymph. Lymphatics may cross the midline and may pass directly through the thoracic wall to the sternal lymph nodes.

Pathology

Dermatohistopathology has developed a specialized vocabulary based largely on human disease. Some of the diagnostic criteria and terminology are not applicable to veterinary pathology

and their use may be confusing if not misleading. The reader is referred to the excellent discussions and illustrations in Hargis (1988), Muller et al. (1983), and Yager and Scott (1985) for guidance in the recognition and diagnosis of conditions that may be encountered in the integument of laboratory dogs. Spontaneous diseases of the skin or the mammary gland are rarely cited, which could reflect either a true absence of pathology or a failure to sample and diagnose minor or incidental lesions.

Nonneoplastic Findings, Spontaneous

Abrasions of the skin commonly result from concrete surfaces and caging. Skin wounds also can be caused by bite wounds during fighting. In addition, wounds may occur as a result of overgrown toenails and interdigital cysts.

Alopecia

Oghiso et al. (1982) reported alopecia of unknown cause associated with atrophy of hair follicles, hyperkeratosis, and necrosis of the epidermis. Alopecia (the complete or partial, diffuse, or circumscribed loss of hair) without underlying skin changes is seen in laboratory dogs over bony pressure points such as the elbow and hock. Continued trauma to such areas may result in a localized hyperkeratotic lesion known as a callus (callosity). Calluses may be ulcerated and infected and become pressure point granulomas.

Circumscribed, erythematous, scaly areas of alopecia near the eyes, commissures of the mouth, or on the forelegs may be a sign of demodectic mange. Demodectic mites in the skin are estimated to occur in 80% of the beagles of some laboratory colonies (Hottendorf and Hirth, 1974). *Demodex canis* is a normal resident of the skin of the dog and inhabits hair follicles and sebaceous glands (Muller et al., 1983; Yager and Scott, 1985). The mites are transmitted by direct contact from dam to nursing puppies. A disease state (demodicosis) develops when the favorable equilibrium between dog and parasite is altered, allowing excessive proliferation of the mite. Microscopic examination of demodectic lesions reveals varying degrees of perifolliculitis, folliculitis, and furunculosis (penetrating or perforating folliculitis) in the presence of a large mite population (Muller et al., 1983).

Dermatosis of the Ear

Multiple irregular, soft tan nodules may be seen on the margin of the pinnae. Microscopically, the lesion consists of orthokeratotic (anuclear) and/or parakeratotic (nucleated) hyperkeratosis and is consistent with a condition in pendulous eared dogs known as ear margin dermatosis (Muller et al., 1983).

Superficial necrolytic dermatitis was reported in a young laboratory beagle with diabetes mellitus. This skin lesion was restricted to the paws and interdigital areas and characterized microscopically by upper epidermal vacuolation of keratinocytes (Yoshida et al., 1996).

Mastitis. Apparently, mammary gland inflammation is rare; a single case of mastitis was found in 499 young beagle bitches (Hottendorf and Hirth, 1974).

Neoplastic Findings, Spontaneous

The peak age for skin neoplasia in the dog is between 6 and 14 years (Muller et al., 1983); however, tumors do occur in young dogs including laboratory beagles. Skin tumors that have been reported in beagles under 2 years of age include one histiocytoma (0.1%) in 647 dogs (Hottendorf and Hirth, 1974) and an unspecified sarcoma and a mast cell sarcoma (Fritz et al., 1966). For a detailed description and classification of canine tumors, the reader is referred to Muller et al. (1983), Moulton (1990), and Meuten (2002). Although mammary tumors are the most common

tumor in bitches, they are rarely encountered in dogs less than 2 years old (Moulton, 1990). A survey of reports on the effects of oral or injectable contraceptives in the beagle revealed that mammary nodules rarely developed in nontreated (control) bitches. In a 7 year study, a total of 40 mammary nodules were palpated in 7 of 18 control bitches (Giles et al., 1978). Most of the nodules were transient and only nine remained in six bitches at the time of necropsy. Histopathological examination of the nine nodules revealed five lobular or intraductal hyperplasias, two benign mixed mammary tumors, and two nonmammary nodules (benign soft tissue tumors, epidermal cysts, lymph nodes, or inflammatory nodules). In a 4-year study, nodules were palpable in 4 of 20 control beagle bitches at 21–24 months (Nelson et al., 1973), but all nodules had disappeared by the time of necropsy. During life span observations of 1343 beagles, 476 (70.8%) of the 672 females and 2 (0.3%) of 671 males had one or more mammary neoplasms (Benjamin et al., 1999; Benjamin, 2001). The reader is referred to Moulton (1990), Misdorp et al. (1999), Goldschmidt et al. (2001), and Misdorp (2002) for descriptions and classifications of tumors and tumorlike lesions of the canine mammary gland.

Musculoskeletal System

Anatomy and Histology

Bones

The vertebrate skeleton supports the body, provides rigid attachment for muscles of locomotion and respiration, protects internal organs, stores minerals, and is the site of blood cell formation (Miller et al., 1967; Wasserman, 1977). The skeleton consists of many bones varying in size, shape, and function. Long bones such as the femur, tibia, humerus, radius, ulna, metacarpals, metatarsals, and phalanges make up most of the appendicular skeleton and serve primarily as levers. The limbs also contain many short bones (carpal and tarsal) that provide flexibility to their respective joints and sesamoid bones that alter the course of tendons and protect tendons at points of greatest friction (Miller et al., 1967). Flat bones are found in both the appendicular skeleton (scapula) and axial skeleton (ribs, sternum, calvarium). The calvarium protects the brain, whereas the ribs and sternum assist in respiration as well as protect the thoracic organs. Irregular bones are characterized by jutting processes. Much of the axial skeleton comprises irregular bones, including the vertebrae, bones of the base of the skull, and the fused hip bones (Miller et al., 1967). The processes of irregular bones are mostly for muscular and ligamentous attachments; however, some are for articulation. The os penis is the single bone of the heterotopic skeleton of the dog and forms the skeleton of the penis. Bones are entirely of mesodermal origin and develop either by direct transformation of connective tissue to bone (intramembranous osteogenesis), by replacement of previously formed cartilage by bone (endochondral osteogenesis), or by a combination of the two processes (Wasserman, 1977). Intramembranous osteogenesis is exemplified by the formation of flat bones of the skull and subperiosteal bone in the shafts of long bones. Endochondral osteogenesis is exemplified by the longitudinal growth of long bones. Bones contain many cell types: endothelial, fat, hemopoietic, chondroblasts, chondrocytes, osteoblasts, osteocytes, and osteoclasts. Of these, osteoblasts, osteocytes, and osteoclasts have specific bone-related functions. The three types of cells are derived from a common ancestor in the bone marrow (Doige, 1988).

Osteoblasts

These arise from bone marrow stromal stem cells to produce and mineralize an organic matrix called osteoid. Osteoblasts are readily observed during the formation of new bone where they

appear as epithelial-like sheets of plump, basophilic cells aligned along bone-forming surfaces. As subsequent rows of osteoblasts differentiate, new osteoblasts begin secreting and osteoblasts of the first row become embedded in bone matrix.

Osteocytes

Osteoblasts buried in mineralized matrix are known as osteocytes. Only about 10% of osteoblasts become osteocytes, the rest apparently die (Jubb et al., 1985). Osteocytes generally appear as single, flat, or oval cells in spaces called lacunae. Although surrounded by bone, osteocytes are not isolated, but maintain contact with other osteocytes and osteoblasts by means of a vast network of cytoplasmic processes contained within minute channels called canaliculi (Doige, 1988). Osteocytes play a role in calcium homeostasis by demineralization and remineralization of perilacunar bone. The demineralizing process is known as osteocytic osteolysis. Osteocytes also retain some capacity for bone formation (Doige, 1988).

Osteoclasts. The typical osteoclast is a large, multinuclear cell with abundant eosinophilic cytoplasm. Osteoclasts arise by fusion of mononuclear phagocytes whose precursors are derived from bone marrow hemopoietic stem cells (Doige, 1988). The principal function of osteoclasts is removal of bone, both matrix and mineral. When active, osteoclasts are located in bone structure concavities known as Howship's lacunae. Osteoclasts are highly mobile, capable of migrating along surfaces of bone and also of entering the bloodstream (Wasserman, 1977). The work of osteoclasts is the basis of the resorption phase of all bone modeling and remodeling (Jubb et al., 1985).

Bone Modeling and Remodeling

Bone modeling refers to the formation and reformation of bone required in the shaping of a growing skeleton (Doige, 1988). Modeling allows the overall shape of bones to be maintained while the skeleton is growing and depends on cellular activity in all parts of the bone. Bone remodeling is the turnover of tissues in mature bones and is the process by which the skeleton renews itself throughout life (Jubb et al., 1985). Remodeling occurs on three surfaces: periosteal, endosteal, and intracortical. Since various bones stop growing at different times, the changeover from modeling to remodeling will vary from site to site in the skeleton. Modeling and remodeling occur in both cancellous (trabecular, spongy) and compact (dense, cortical, Haversian) bones.

Cancellous bone. This is elaborated in the extremities of long bones (epiphysis and metaphysis), forms the internal substance of short and irregular bones, and is interposed between the cortices of most flat bones (Miller et al., 1967). The trabeculae of cancellous bone vary in form from densely packed plates to interconnecting rods to a delicate filigree (Jee et al., 1970). No cancellous bone is present in the middle region of long bones and the space thus formed is known as the medullary cavity. The medullary cavity of long bones in adult animals is largely filled with yellow bone marrow. The cancellous bone of ribs and vertebrae and many short and flat bones is filled with red marrow throughout life (Miller et al., 1967).

Compact Bone

This comprises mostly long, bony rods with a central vascular canal. This structure is known as the osteon, or Haversian system, and is the classic structural unit of diaphyseal bone. Osteons form between trabeculae of cancellous bone, eventually filling in the intertrabecular space and converting cancellous bone to compact bone (Jubb et al., 1985). The remaining interstices of cancellous bone are occupied by red marrow.

Joints

Joints, or articulations, are structures that join two or more bones in unions that may or may not be movable. Three main types of joints are recognized: fibrous, cartilaginous, and synovial.

Fibrous Joints

These are simple unions that provide for little movement. Examples are sutures of the skull and unions of long bones such as the tibia and fibula (tibiofibular syndesmosis).

Cartilaginous joints. Two types of cartilaginous joints are recognized: hyaline and fibrocartilaginous.

Hyaline Cartilaginous Joints

The site of endochondral osteogenesis in long bones (physis) is a temporary hyaline cartilage joint that is eventually replaced by bone. The unions between ribs and costal cartilages are permanent hyaline cartilage joints, although they may be partially ossified with age (Miller et al., 1967).

Fibrocartilaginous Joints

The unions between the right and left mandibular bodies (mandibular symphysis) and right and left os coxae (pelvic symphysis) are examples of fibrocartilaginous joints. The mandibular symphysis persists throughout life, but the pelvic symphysis ossifies in the adult (Miller et al., 1967). The unions of vertebrae (intervertebral disks) are special fibrocartilaginous joints consisting of a collagenous ring (annulus fibrosus) surrounding a space filled with semifluid material (nucleus pulposus).

Synovial Joints

These joints are the truly movable articulations. All synovial joints feature a capsule, cartilaginous articular surfaces, joint cavity, and a lubricating fluid. A few synovial joints also possess intra-articular ligaments, fibrocartilaginous plates (menisci), and fat pads (Miller et al., 1967). The fibrous joint capsule is continuous with the periosteum of the bone ends and encloses the joint cavity. The inner layer (intima) of the joint capsule lines the cavity except on the surfaces of the cartilage and is responsible for the production and turnover of the fluid (synovia) that lubricates the joint and nourishes the articular surfaces (Jubb et al., 1985). The articular surfaces of all principal synovial joints are covered with typical hyaline cartilage that is thickest in the young animal and at sites of maximum weight bearing. The normal capsule is strong but not rigid. Normal intima is smooth and glistening. The articular cartilage in young and healthy animals is smooth, white or somewhat bluish, semitransparent, and moist. The normal synovia is viscous, clear, colorless, or slightly yellow (Jubb et al., 1985; Doige, 1988).

Muscle

Muscles are specialized collections of cells that produce directed movement through strong organized contractions (Venable and Dellmann, 1976). Muscles are customarily classified as smooth, cardiac, and skeletal.

Smooth Muscle

This type of muscle is found in the walls of the digestive, respiratory, urinary, and reproductive tracts, blood vessels, spleen, and eyeball, and it is also associated with glands and hair follicles

(Miller et al., 1967; Goll et al., 1977). Smooth muscle has a simple appearance consisting of spindle-shaped (tapered) cells without cytoplasmic cross striations and a single, centrally placed nucleus. Smooth muscle is commonly referred to as visceral muscle (by its location) or involuntary muscle (by its innervation) and has also been called plain, or unstriated, muscle (Miller et al., 1967).

Cardiac Muscle

This type of muscle forms the bulk of the heart and combines the features of smooth and skeletal muscles. As in smooth muscle, the nuclei are centrally placed and the fibers are under involuntary nervous control. As in skeletal muscle, the fibers are cross striated and multinucleated. Cardiac muscle has also been called involuntary striated muscle.

Skeletal Muscle

This type of muscle is so named because of its relationship to the skeleton. Skeletal muscle comprises the single largest tissue mass in the body; 40% of the body weight for mammals in general (Goll et al., 1977) and from 35% to 54% of the body weight of beagles (Andersen and Goldman, 1970). Skeletal muscles range in size from the minute stapedius muscle of the middle ear to the massive muscles of the rump (Miller et al., 1967). Individual muscles are surrounded by and separated from other muscles by a sheet of connective tissue known as the epimysium (from Greek epi meaning upon, above, beside). The epimysium may be fairly thick and tough and is the site of intermuscular fat deposits. At irregular intervals, thinner sheets of connective tissue, called perimysium (Greek pen around), pass into the muscle and divide it into bundles (fasciculi). The perimysium also envelopes blood vessels and nerves and is the site of intramuscular fat deposits (Goll et al., 1977). Very delicate sheets of connective tissue, called endomysium (Greek "endo" = "within"), extend from the perimysium and surround individual muscle fibers. The endomysium lies immediately adjacent to the muscle cell outer membrane (sarcolemma). The endomysium carries a longitudinally oriented capillary network. Skeletal muscle fibers are striated and multinucleated, with the majority of nuclei located peripherally. Skeletal muscle is under control of the somatic or voluntary nervous system and hence is also called somatic, or voluntary, muscle.

Muscle fibers. Not all normal skeletal muscles have the same depth of color. Color variation depends upon a number of factors but is in part due to the relative percentages of type I and type II fibers. Histochemical techniques have allowed the division of skeletal muscle fibers into two major groups: type I fibers, which are rich in oxidative enzymes and darker in color and show a slow twitch response, and type II fibers, which are rich in glycogen and paler in color and show a fast twitch response (Hulland, 1985). The ratio of the two fiber types varies widely from muscle to muscle. In the dog, type I fibers comprise less than 15% of the extensor carpi radialis and over 90% of the vastus intermedius (Armstrong et al., 1982).

Necropsy and Laboratory Techniques

A "complete" postmortem examination rarely includes a complete examination of bones, joints, and muscles. While a complete dissection of the musculoskeletal system is neither practical nor necessary, something more than a cursory examination is in order. Antemortem clinical findings, including clinical laboratory results, should be known. A brief visual examination and palpation of the body before exsanguination or skinning should reveal swollen or stiff joints, muscle wasting, and skeletal deformities and asymmetry. Certain bones, joints, and muscles should be routinely examined to provide completeness to the necropsy and to establish normal baseline values for color, hardness, size, volume, etc. The prosector should be alert to general changes in the musculoskeletal system during the course of the postmortem examination. Ventral and lateral muscles of the neck

and trunk are exposed during the primary ventral midline incision and reflection of the skin. Skin should also be reflected from the inner aspect of the limbs to expose the shoulder, elbow, and knee joints and major muscles of the legs. Several of the large medial muscles of the limbs must be cut to fully extend the legs, and in the process, large cross-sectional areas of muscle are brought to view. Several large synovial joints should be opened completely. The hindlimbs can be extended fully only by disarticulating the coxofemoral joints; however, in the process, the joints are usually contaminated with blood from the femoral vessels. With practice, the shoulder, elbow, and knee joints may be opened without contamination, but the disarticulation must be done carefully to avoid slicing articular cartilage. Joints should be examined immediately upon opening because articular cartilage rapidly dehydrates and discolors when exposed to air (Doige, 1988). It is helpful to compare contralateral joints; however, the prosector should realize that under normal circumstances the synovial fluid may have a different appearance from joint to joint within an animal and in the same joint between animals (Jubb et al., 1985). An additional opportunity to examine a large synovial joint arises when the atlanto-occipital joint is disarticulated to remove the head.

During removal of the spinal cord (as a whole or in segments), the surface of the spinal canal overlying intervertebral disks should be examined. The ventral surface of the vertebral column should be palpated following removal of the thoracic, abdominal, and pelvic viscera. Bone strength may be assessed as the ribs, calvarium, and os coxae are cut while opening the thoracic, cranial, and pelvic cavities. Jaws and teeth should be examined during removal of the tongue and larynx. At least one long bone (preferably a femur) should be examined in detail and fixed for microscopic evaluation. The femur should be cut in a standardized, midline longitudinal plane to establish a baseline for normal width of cortical bone, density of cancellous bone, width and uniformity of physes, curvature of articular surfaces, and relative proportion of red and yellow marrow. The marrow contents may be flushed away by a jet of water from one of the halves for better exposure of bony structure.

For good fixation, bone slabs should be no wider than 5 mm; therefore, additional bone will need to be cut from the proximal and distal ends of the femur. The femur is collected primarily for the study of osseous, cartilaginous, and articular tissues. An additional bone, preferably a flat bone such as a sternebra, should be collected specifically for bone marrow cytology. The sternebrae of young dogs may be cut with a stout sharp knife, thus avoiding the problem of the marrow surface being filled with bone and muscle debris from a saw.

Skeletal muscle is usually one of the last tissues collected at necropsy. Delay in fixation is not a concern, for muscle is one tissue that may appear worse histologically when fixed immediately than when fixed hours, even days, after death (McGavin, 1983). Artifacts are the problem. Fresh muscle is very sensitive and will vigorously contract when pinched, crushed, cut, stretched, and placed in most fixatives. The resulting artifacts (severe contraction bands; sarcoplasmic masses; shredded, cracked, and rounded and hyalinized fibers) will mask and even mimic pathological change (McGavin, 1983).

Another problem in interpretation confronts the microscopist when the histological sections contain mostly tangentially sectioned fibers. Both transverse and longitudinal sections of the muscle are required for the proper interpretation of many pathological processes. Transverse and longitudinal sections can be obtained only from muscles in which the majority of the fibers are oriented parallel to each other. Three hindlimb muscles (biceps femoris, semitendinosus, semimembranosus) have such an orientation of fibers and are also large (for ease of sampling) and easily identified. In addition, each of the aforementioned muscles presents a fairly good representation of type I and type H fibers. The following percentages of type I fibers were found by Armstrong et al. (1982): biceps femoris (32 ± 8), semitendinosus (27 ± 11), and semimembranosus (28 ± 5).

Once selected, the same muscle should be the site from which all samples are taken. To minimize the problem of artifacts, the muscle must be handled gently; but, most importantly, the muscle must be prevented from contracting during removal and fixation. McGavin (1983) recommends the following relatively simple procedure: make two parallel incisions, 3–6 mm apart, into the belly of

the muscle and, in the same direction as the muscle fibers, suture each end of the isolated strap of muscle to a narrow, flat wooden stick, undercut the muscle, sever the muscle beyond the sutures, remove the stick and muscle together, and place in fixative.

Paraffin embedded, H&E stained sections of bone and muscle meet the needs of most toxicology studies. There are, however, a number of special laboratory techniques to study both bone and muscle. Many of the bone procedures have been used extensively in the beagle and include the measurement of bone ash, volume, and specific gravity; labeling sites of mineralization by fluorescent markers; and microscopic and microradiographic examination of undemineralized sections (Saville and Krook, 1969; Jee et al., 1970; Anderson and Danylchuk, 1978, 1979a–c; Jorch and Anderson, 1980; Martin et al., 1981; Kunkle et al., 1982; Norrdin and Shih, 1983; Snow et al., 1986). Bone specimens for routine H&E staining should be well fixed, free of debris from the bone saw, and not overly decalcified and represent both cancellous and cortical bone, an articular surface, and a physis. McGavin (1983) lists special stains and enzyme histochemical procedures to study the muscle. As mentioned, H&E stained specimens of muscle should always include transverse and longitudinal sections.

Pathology

Few spontaneous findings are described for the musculoskeletal system. Hottendorf and Hirth (1974) report a fractured rib in 1 (0.1%) of 1000 dogs and chondrodystrophic changes in the costochondral junctions of 1 (0.1%) of 647 dogs. Hottendorf and Hirth (1974) also report congenital hernias (digestive system). In the writers' experience, these are usually small umbilical hernias containing only omental fat. Congenital hernias were observed grossly in 5 (0.5%) of 1000 dogs. Barron and Saunders (1966) report the fortuitous finding of *Toxocara* granulomas in skeletal muscle.

Digestive System

Anatomy and Histology

The digestive system consists of a series of connecting, mostly tubular, hollow structures that includes the mouth, pharynx, esophagus, stomach, and small and large intestines and a group of accessory organs that includes the teeth, tongue, salivary glands, liver, gallbladder, and pancreas. The tubular portion from the esophagus distally is the alimentary canal.

Mouth

The mouth (oral cavity) is the most anterior opening and cavity of the digestive system. Its limits anteriorly and anterolaterally are the upper and lower lips, posterolaterally the right and left cheeks, dorsally the palate, and ventrally the floor of the mouth and tongue. Anteriorly, the palate has a bony core and is termed the hard palate. Posteriorly, the palate has a muscular core and is termed the soft palate. The soft palate is unusually long in the dog and may extend to or beyond the epiglottis (Miller et al., 1967). The gingiva is the keratinized epithelial membrane that covers the alveolar processes of the jaws and attaches to the teeth. The gingival tissue adjacent to the tooth surface is known as the free gingival margin. The dog usually has 42 permanent teeth (Miller et al., 1967), whose names and placement in the upper and lower dental arcades are shown in the following formula (I, incisor; C, canine; PM, premolar; and M, molar):

$$1\ 3/3\ C\ 1/1\ PM\ 4/4,\ M\ 2/3 \times 2 = 42$$

According to their location, the incisors are known as central, intermediate, or comer; the premolars as first, second, third, or fourth; and the molars as first, second, or third. Deciduous (temporary)

teeth immediately replace permanent teeth in the dog (Miller et al., 1967). In the beagle, the average age of eruption (in months) for permanent teeth is as follows (Bartley et al., 1970):

	Central	Intermediate	Comer	
Incisor	3.8	4.1	4.4	
	3.9	4.1	4.1	
Canine	4.7			
	4.7			
	First	**Second**	**Third**	**Fourth**
Premolar	3.5	5.0	5.1	4.5
	4.3	5.0	5.2	5.2
Molar	4.4	5.2	—	—
	4.3	5.0	5.8	—

Permanent dentition should be complete in most beagles by the age they are placed on study.

The tongue forms most of the floor of the mouth. The posterior one-third of the tongue is the root and the slender anterior two-thirds is the body. A long mucosal fold (frenulum) connects the body of the tongue to the floor of the mouth.

Pharynx

The mouth is continuous posteriorly with the pharynx, a funnel-shaped connection between the mouth and the esophagus and between the nasal cavity and the larynx. The pharynx serves both digestive and respiratory functions. An exclusively respiratory portion (nasal pharynx) lies above the soft palate. The portion below the soft palate (oral pharynx) serves a mixed digestive/respiratory function, being respiratory during panting.

The nasal and oral pharynges unite just posterior to the soft palate to form the pharyngeal isthmus, where the digestive and respiratory tracts change relationships. The respiratory tract continues ventrally as the larynx and thereafter as the trachea. The digestive system continues dorsally as the laryngeal pharynx and thereafter as the esophagus. The lateral walls of the nasal pharynx are obliquely pierced by two slit-like openings, the pharyngeal ostia of the auditory tubes. The lateral walls of the nasal pharynx are indented by two crypts that contain long, thin lymphoid structures, the palatine tonsils.

Salivary Glands

Salivary glands are the first of the accessory organs to discharge secretions into the digestive tube. Salivary glands are classified by size (major, minor) and by secretion (mucous, serous, mixed) and named by location. All major salivary glands are paired and include the parotid, mandibular, sublingual, and zygomatic glands (Miller et al., 1967).

Saliva

The individual and collective secretions of the salivary glands are discharged into both the dorsal and ventral parts of the mouth. Dorsally, most secretions come from the parotid and zygomatic glands and ventrally from the mandibular and sublingual glands. The saliva of the dog has no enzymatic activity of note (Harvey et al., 1983).

Parotid Gland

This is a coarsely lobulated and reddish V-shaped organ that closely embraces the base of the ear. It is predominantly serous, but occasional isolated mucous secretory units may be found (Stinson and Calhoun, 1976). The parotid duct opens into the mouth lateral to the upper fourth premolar tooth.

Mandibular Gland

This is a lightly lobulated and light tan ovoid body lying just caudal and ventral to the parotid gland. It is a mixed gland (Stinson and Calhoun, 1976) and its ducts open below the tongue near the frenulum.

Sublingual Gland

This gland consists of a series of two or more elongated, lobulated masses extending from the mandibular gland to the anterior margin of the frenulum. The most posterior portion of the sublingual gland is enveloped by the capsule of the mandibular gland (Miller et al., 1967). Grossly, the sublingual gland is distinguished from the mandibular gland by its darker color; however, its subcapsular location within the mandibular gland may puzzle the microscopist when first seen. The sublingual gland is a mixed gland (Stinson and Calhoun, 1976) whose ducts empty onto the floor of the mouth.

Zygomatic Gland

This gland is a moderately lobulated gland located beneath the zygomatic arch, ventral and posterior to the eye. The zygomatic gland is predominantly mucous, but a few serous demilunes are present (Stinson and Calhoun, 1976). Zygomatic gland secretions enter the mouth through several openings lateral to the upper molar teeth.

Minor Salivary Glands

These glands consist of clusters of serous, seromucous, or mucous elements found in various oral structures, including the tongue, lips, palate, and pharynx.

Esophagus

The esophagus is the first part of the alimentary canal and connects the laryngeal pharynx with the stomach. The esophagus may be divided into cervical, thoracic, and abdominal segments. The cervical esophagus extends from the pharynx to the thoracic inlet, the thoracic esophagus extends from the thoracic inlet to the diaphragm, and the abdominal esophagus is the short segment between the diaphragm and the stomach. The abdominal esophagus acts as a flutter valve and is important in preventing gastroesophageal reflux (Strombeck, 1979).

The esophagus is capable of great distention except at its beginning and end and as it passes through the thoracic inlet. The collapsed mucosa forms numerous longitudinal folds. In the dog, the entire length of the mucosa is covered by nonkeratinized stratified squamous epithelium. The submucosa contains numerous mucous glands with serous demilunes, and the tunica muscularis is composed entirely of two oblique layers of striated muscle fibers (Miller et al., 1967).

Stomach

The stomach is the largest dilatation of the alimentary canal. A line drawn through the axis of the stomach appears somewhat like a letter C. The longer, outer convex surface of the organ is the greater curvature. The shorter, inner concave surface is the lesser curvature. The walls between the two curvatures are the dorsal and ventral walls. The inlet from the esophagus is the cardiac ostium and the outlet into the small intestine is the pyloric ostium.* For gross, microscopic, and functional purposes, the stomach may be divided into five, three, and two regions, respectively (Strombeck, 1979). Grossly, the regions consist of a narrow zone between the esophagus and body known as the cardiac region. The body is the large middle region, and the fundic region is the blind outpocketing of the body located to the side of the cardia on the greater curvature. The terminal, funnel-shaped, one-third of the stomach is the pyloric region. The pyloric region is subdivided into an initial thin-walled portion (pyloric antrum) and a terminal thick-walled portion (pyloric canal). The normal color of the cardiac, fundic, and body mucosa is pink to grayish red. The pyloric mucosa is a pale tan. The mucosa of an empty or even moderately distended stomach is thrown into numerous folds (gastric rugae).

Close examination of the gastric mucosa reveals that it is composed of minute raised areas (areae gastricae) surrounded by narrow furrows. This blocklilce subdivision of the gastric mucosa may be recognized in tissue section, especially in the pyloric region. The gastric mucosa in the dog is entirely glandular.

According to its glandular makeup, the stomach is histologically divided into cardiac, gastric gland proper, and pyloric regions (there is intermixing of glands between adjacent regions). The cardiac and pyloric regions comprise primarily mucous glands. Cardiac glands are characterized by deep gland openings (foveolae) and short gland bodies. Pyloric glands have foveolae and bodies of about equal length. The body and fundic regions are populated principally by gastric glands proper (sometimes erroneously called fundic glands), which have comparatively shallow foveolae and long bodies. The gland bodies are lined by mucous neck, parietal (oxyntic), chief, and argentaffin cells. Small numbers of parietal and argentaffin cells are also found in the pyloric region and parietal cells also may be found in the cardiac region. The submucosal mucous glands of the esophagus extend into the cardiac region (Stinson and Calhoun, 1976). Similarly, the submucosal glands of the duodenum (Brunner's glands) extend into the submucosa of the pyloric region.

Functionally, the proximal two-thirds of the stomach (body and fundus) adapts by expansion to serve as a storage reservoir and the caudal one-third (pyloric region) performs as a grinding mill and funnel to propel ingesta into the small intestine (Strombeck, 1979). Studies of the rate at which digesta move through the gastrointestinal tract of the dog reveal that at 8 hours, roughly 90% of the meal is in the stomach and roughly 10% has entered the small intestine (Stevens, 1977). At 12 hours, approximately equal quantities are found in the stomach, small intestine, and colon. At 24 hours, roughly 30% remains in the stomach, 10% is in the small intestine, and 30% each in the colon and feces. The bulk of the meal (90%) has been evacuated at 38 hours.

Small Intestine

The small intestine extends from the stomach to the colon. It is the longest portion of the alimentary canal, representing about 85% of length of the entire intestine (Stevens, 1977). (A length of 225–290 cm has been reported for the small intestine of the adult beagle; Andersen, 1970.)

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* The term "cardia" has not been used because it has variously referred to the cardiac region, ostium, and sphincter. Similarly, the term "pylorus" was not used because it has variously referred to the pyloric region, antrum, canal, ostium, and sphincter.

The small intestine represents only 23% of total capacity of gastrointestinal tract. The stomach, cecum, colon, and rectum represent 62%, 1%, and 13%, respectively (Stevens, 1977).

There are no gross features to definitely separate the three segments of the small intestine, and the beginning (duodenum), middle (jejunum), and end (ileum) must be set arbitrarily.

Duodenum. This is considered to be the proximal one-tenth, or 25 cm, of the small intestine (Miller et al., 1967). The duodenum is divided into cranial, descending, caudal, and ascending portions. Brunner's glands are limited primarily to a narrow region of the pyloric duodenal junction (Titkemeyer and Calhoun, 1955). Pancreatic and bile ducts discharge into the descending duodenum. The common bile and ventral pancreatic ducts share a small protuberance (major duodenal papilla) found 3–5 cm caudal to the pyloric ostium. The dorsal pancreatic duct opens on a smaller protuberance (minor duodenal papilla) located about 5 cm caudal to the major papilla. To the uninitiated, the duodenal papillae may appear to be tumorous enlargements in the gut wall. While the pancreatic ducts penetrate the gut wall more or less at right angles, the common bile duct courses intramurally for a distance of 1.5–2.0 cm. The microscopist should be aware of the lengthy intramural placement of the bile duct and not misinterpret its presence as an anomaly or tumor.

Jejunum and Ileum

These comprise the bulk of the small intestine. Most veterinary anatomists consider the jejunum to be substantially longer than the ileum and generally regard only the short, usually contracted, terminal part of the small intestine as ileum (Miller et al., 1967). The circularly arranged folds (plicae circulares) that characterize the small intestine of humans and some domestic animals are absent in the dog. The intestinal glands (crypts of Lieberkühn) are long in the dog, resulting in villus length: crypt-depth ratios of 2:1, 1:1, or even less (compare to ratios of 4:1 or greater in species such as mice, rats, pigs, and humans). Dogs lack Paneth cells (Stinson and Calhoun, 1976).

Aggregated lymph follicles (Peyer's patches) are easily recognized in the dog from either the exterior or interior surface of the small intestine. Generally, the patches appear on either side of the mesenteric attachment. On the mucosal surface, Peyer's patches appear as ovoid elevations measuring about 1.5 by 2.0 cm. An average of 22 Peyer's patches have been reported for the dog (Titkemeyer and Calhoun, 1955). They exist throughout the length of the small intestine but most are found in the duodenum and jejunum. Villi overlying Peyer's patches are generally short and oddly shaped, may even be entirely effaced, and in general are not representative of villi elsewhere in the gut. Peyer's patches, solitary lymph follicles, tonsils, and the diffuse lymphoreticular tissue of the digestive system comprise the gut-associated lymphoid tissue (GALT).

Large Intestine

The large intestine of the dog is short, representing about 13% of entire length of the intestine (Stevens, 1977), and it lacks special features such as sacculations, bands, and a vermiform appendix found in other species. The large intestine begins at the ileocolic orifice and ends at the anus and includes the cecum, colon, rectum, and anal canal.

Colon. This is the most proximal segment of the large intestine. It is about 25 cm long and is divided into ascending, transverse, and descending portions. The cecum is a relatively short and small diverticulum of the ascending colon and communicates with the colon only through the cecocolic orifice. (Some authors erroneously refer to an ileocecal valve and orifice.) The descending colon is the longest and straightest portion of the colon. The large intestine continues as the short (about 5 cm long), entirely intrapelvic portion known as the rectum. The anal canal is the terminal 1 cm of the alimentary tube. For its length, the anal canal is very complex with three mucosal zones, two types of glands, and the site of drainage for the anal sacs. Solitary lymph nodules are found throughout the mucosa of the entire large intestine; however, they tend to be particularly large in the

rectum and often appear as raised nodules, 3–4 mm in diameter, with central craterlike depressions. The depressions are due to circular discontinuities in the muscularis mucosae, allowing intimate mixing of intestinal glands and submucosal lymphoid aggregates. Solitary lymph nodules are also found in the esophagus, stomach, small intestine, and gallbladder mucosae.

Liver

The liver is undoubtedly the digestive organ of greatest interest in toxicology studies. It is a large organ in keeping with its many functions. The liver represents about 7% of the body weight of 2–3-month-old pups and about 4% in adults (Andersen, 1970). The liver is divided into four lobes: left, right, quadrate, and caudate. The left lobe is the largest, comprising up to one-half of the entire liver. Both the left and right lobes are divided into sublobes, which are called lobes nevertheless. On the left, the larger sublobe is the left lateral lobe. Medial to it lies the left medial lobe. The right hepatic lobe is divided into the right medial lobe and the right lateral lobe. The quadrate lobe lies between the right and left hepatic lobes. The gallbladder lies partially in a depression (fossa) on the right side of the base of the quadrate lobe. The left half of the fossa is formed by the base of the right medial lobe. The caudate lobe is the most irregularly shaped and most caudally placed lobe of the liver. Its most cranial portion is called the papillary process. The most caudal portion is the caudate process, which is marked by a deep impression for the right kidney. The fresh liver is reddish brown, firm to the touch, yet friable. Normally, the borders (margins) are sharp-edged and the cut surface does not bulge. The capsular surface is patched tightly covered by a thin, transparent membrane (peritoneum). Close inspection reveals about 1 mm sized subunits (hepatic lobules) that vary considerably in prominence according to the physiological and pathological state of the liver.

Gallbladder

The gallbladder is a pear-shaped vesicle that lies between the quadrate and right medial lobes of the liver. It has a capacity of 10–15 mL (Andersen, 1970). When fully distended, the gallbladder is visible on the diaphragmatic surface of the liver. The emptying of the gallbladder is related to gastric digestion; during fasting, it remains distended. The gallbladder has a neck, body, and a blind, rounded, cranial end known as the fundus. Bile is formed in the liver and stored and concentrated in the gallbladder. The dog has a high concentrating ability (Stevens, 1977). Bile reaches the gallbladder by way of the hepatic and cystic ducts. The cystic duct extends from the neck of the gallbladder to the first bepatic duct. The common bile duct extends from the junction of cystic and hepatic ducts to the major duodenal papilla. Bile may be watery to mucoid in consistency and greenish yellow to golden brown in color. Dark green flecks are not unusual. The gallbladder mucosa is normally smooth but solitary lymph follicles may cause a slight roughness.

Pancreas

The pancreas is a V-shaped gland formed by the fusion of a slim right lobe and a shorter, but thicker and wider left lobe. The left lobe lies in the greater omentum adjacent to the stomach. The right lobe lies in the mesoduodenum adjacent to the descending duodenum. Numerous variations in the pancreatic duct system have been described in the dog (Miller et al., 1967). Most commonly, the larger ventral pancreatic duct drains the right lobe and the smaller dorsal pancreatic duct drains the left lobe. The ventral pancreatic duct terminates as a slit-like opening adjacent to the common bile duct on the major duodenal papilla. The dorsal pancreatic duct opens upon the minor duodenal papilla. The pancreas is soft and coarsely lobulated and has a pinkish-gray coloration in life. The pancreatic islets of the dog usually are not visible to the naked eye.

Necropsy and Laboratory Techniques

Disease of the digestive system produces a wide variety of clinical signs. Some signs are non-specific (anorexia, weight loss, pain, weakness, reluctance to move, generalized malaise, shivering, fever, dehydration, hemorrhage, anemia), and some signs are shared with disease of other organs (depression, coma, polydipsia, polyuria), but many signs indicate disease somewhere within the digestive system.

The most common signs associated with problems in the upper digestive tract (mouth, pharynx, esophagus, stomach, proximal small intestine) are vomiting (forcible expulsion of ingesta into the mouth), regurgitation (passive backward flow of ingesta into the mouth or nasal cavity), and dysphagia (difficulty in swallowing) (Strombeck, 1979). Additional signs of upper digestive tract disease are halitosis (offensive breath), excessive salivation, hematemesis (blood in the vomitus), melena (black feces, digested blood), abdominal pain, and bloating.

Diarrhea (an increase in the frequency, fluidity, or volume of feces) is the most consistent indicator of problems in the lower digestive tract (middle and distal small intestine, large intestine) (Strombeck, 1979). The character of the diarrhea (and other signs) often indicate what part of the intestine is diseased.

Signs of small intestine involvement include large quantity of bulky or watery feces, undigested food in the feces, melena, weight loss, and generalized malaise (Sherding, 1983). Signs of large intestine involvement include very frequent defecation, small volume of feces, urgency, tenesmus (painful straining), mucus in the feces, red-stained feces (fresh blood), and constipation.

Liver disease is also associated with numerous clinical signs, including abdominal pain, anorexia, ascites, coma, depression, dark-brown urine, dark- or light-colored stools, diarrhea, fever, hemorrhage, icterus, polydipsia (excessive thirst), polyuria (abnormally large volume of urine), pruritus, weakness, weight loss, and vomiting (Strombeck, 1979). Since the liver has a large functional reserve and an amazing capacity to regenerate, as much as 70%–80% of the liver's mass must be impaired before signs of dysfunction are seen (Hardy, 1983).

The clinical signs of acute pancreatic disease include vomiting, pain, anorexia, depression, fever, diarrhea, and abdominal distress (aside from pain, dehydration, shock, and respiratory distress) (Strombeck, 1979). Signs of chronic pancreatic disease (pancreatic atrophy) include diarrhea, steatorrhea (excessive amount of fat in the feces), and weight loss. With these signs in mind and all available clinical and laboratory data at hand, the prosector may undertake examination of the digestive system.

The oral cavity should always be examined, preferably after anesthesia and before exsanguination. The breath of a healthy dog is usually not unpleasant. The normal oral mucosa is pink, has a smooth and glistening surface, and shows little accumulation of saliva (Harvey et al., 1983). Normal free gingival margins may be slightly hyperemic. The oral cavity may be fully explored after removal of the tongue and pharynx. A cross section of the body of the tongue is usually taken to represent the oral mucosa histologically. The parotid and mandibular salivary glands should be inspected. The mandibular gland is usually chosen to histologically represent the salivary gland system. The mandibular gland is a reasonable choice for it is readily accessible and contains both mucous and serous secretory units. The initial examination of the thoracic and abdominal viscera should be done with all organs in place and natural relationships undisturbed. This is particularly true when a dilated, flaccid esophagus is found (megaesophagus), for one possible cause is a persistent right aortic arch. The tongue, pharynx, larynx, esophagus, and trachea are usually removed intact along with the heart, great vessels, thymus, and lungs. The esophagus should be completely opened and examined and a midsection collected for histopathology. The pancreas is excised (after removal of the spleen and liver) and examined and a specimen taken (consistently from the right or left lobe) for histopathology. The gastrointestinal tract is removed.

(The pelvic girdle must be cut through at the obturator foramina to excise the rectum and anal canal.) The suspensory ligaments, omenta, and mesentery may be stripped from the gastrointestinal tract as it is removed. The tract should be completely opened and the entire length examined. The stomach unfolds nicely when opened along the greater curvature. Bulk digesta and feces may be picked from the surface. Brief flushing with tap water may be required to remove adherent material. Sloshing a specimen in saline or fixative may be desirable for critical examination of lesions. A complete sampling of the gastrointestinal tract would include two samples of the stomach (body and pyloric regions), cranial duodenum (to include Brunner's glands), jejunum (midportion), ileum (a few centimeters from the ileocolic junction), entire cecum, and a segment of the descending colon and rectum. One of the jejunum/ileum specimens should include a Peyer's patch.

All of the specimens (except the cecum) are preferably fixed in a flattened position. This may be done by stapling the specimens (serosal surface down) to small pieces of corkboard. The boards may be labeled to identify the specimens. The gallbladder also should be fixed in a flattened position. All surfaces of the liver should be examined and multiple parallel slices made through each lobe. Slices of tissue (no wider than 5 mm) should be taken from at least two lobes for histopathology. The right and left lateral lobes are suggested.

Pathology

Numerous observations have been reported for the digestive system of young laboratory beagles. Most of the observations are of no clinical or experimental significance. A few may complicate interpretation of apparent treatment-related effects and the pathogenesis of several have never been explained.

Mouth and Salivary Glands

Changes in the general health may be evident during examinations in the oral mucosa. Anemia results in pale mucous membranes. Icterus (jaundice) results in a yellowish appearance of the mucous membranes associated with circulating bile pigments. Icterus can be caused by excessive hemorrhage, lysis of red blood cells (hemolysis), gallbladder or bile duct obstruction, and liver diseases involving the bile ducts. Oral papillomas (papillomatosis or warts) are benign epithelial tumors caused by papovaviruses. They occur mainly in young dogs and can spontaneously disappear. The warts may be found on the lips, inside the cheeks, and on the tongue, palate, and pharynx. The gums are usually not affected. Oral papillomas were observed grossly in 2 (0.2%) of 1000 dogs (Hottendorf and Hirth, 1974).

Teeth and gums. A number of minor dental abnormalities may be encountered such as missing permanent teeth (usually upper and lower premolars), retained deciduous teeth (usually canine), imperfect apposition of teeth, dental plaque (soft bacterial masses), and dental calculus or tartar (mineralized plaque, usually discolored, most abundant next to orifices of salivary ducts). Inflammation of the gums (gingivitis) is often associated with plaque or tartar around the teeth.

Tongue. In addition to oral papillomatosis affecting tongue, inflammation of the tongue (glossitis) was an infrequently observed lesion. Glossitis was reported in 1%–2% of young dogs (Glaister, 1986). Granulomatous inflammation can result from fragments of sawdust bedding embedded in the tongue.

Salivary glands. Inflammation (sialadenitis) is common with mild focal sialadenitis reported in 35 (5%) of 647 dogs (Hottendorf and Hirth, 1974). Focal fibrosis with atrophy of acini can be seen frequently, particularly in the parotid gland; occasionally, an increase in mucous glands can be seen in the parotid glands (Sato et al., 2012).

Esophagus

Megaesophagus (grossly dilated and flaccid esophagus) may be seen. This is a congenital condition occasionally due to a persistent right aortic arch, but more commonly due to an apparent neuromuscular developmental disorder.

Esophageal hypertrophy is characterized by a swollen, thick-walled esophagus that suggests reflux esophagitis. Inflammation of the esophagus (reflux esophagitis) can occur as an erosive and ulcerative lesion of the esophageal wall in dogs with histories of repeated regurgitation or vomiting. Slight dilatation of esophageal gland ducts may be seen in an otherwise normal esophagus. Occasionally, atrophy or hypertrophy of the esophageal gland can be seen (Sato et al., 2012).

Stomach

Chronic inflammation (gastritis) is a common finding with 58 (9%) observed in 647 dogs (Hottendorf and Hirth, 1974). Granulomatous inflammation was uncommon with 1 (0.1%) in 647 dogs as reported by Hottendorf and Hirth (1974). Gastric glands may be slightly dilated. Microscopic mineralization (microcalculi), which occurs as basophilic granules in the gastric mucosa, was reported in 7%–10% of young beagles by Glaister (1986). The prevalence of Helicobacter-like organisms is very high and reaches 100% among laboratory beagle dogs. A variety of *Helicobacter* species of bacteria have been reported to infect the stomach of dogs including *H. felis*, *H. bizzozeronii*, *H. salmonis*, *H. (Flexispira) rappini*, *H. bilis*, *H. heilmannii*, and *H. cyanogastricus* (Neiger and Simpson, 2000; Lanzoni et al., 2011). *H.* spp. are generally seen in the surface mucus, gastric glands, and parietal cells and occur in gastric regions (Lanzoni et al., 2011). *H.* spp. infection can result in mild chronic inflammation characterized by infiltrations of small numbers of lymphocytes and plasma cells generally toward the deeper parts of the mucosa (Lanzoni et al., 2011). Experimental infection of gnotobiotic beagle dogs with *H. felis* resulted in large numbers of lymphoid follicles in the gastric mucosa (Lee et al., 1992; Simpson et al., 1999). Lymphocytic nodules or follicles may be present within the lamina propria, particularly of the pyloric region.

Intestine

Congestion of blood vessels in the gastrointestinal mucosa is common. This congestion can result from digestive processes but can also be secondary to inflammation and restricted blood flow or occur as agonal changes.

The presence of ectopic tissue is occasionally noted in the intestines and includes ectopic pancreatic tissue in the submucosa near the duodenal papilla and ectopic fundic gland in the jejunum (Sato et al., 2012).

Dilatation of the crypts is most frequently observed in the duodenum and less frequently in other sections of the small and large intestines (Sato et al., 2012). Dilated crypts often contain inflammatory cell infiltrates. Inflammation of the large (cecitis and colitis) and small (enteritis) intestines was very common. Catarrhal enteritis was reported in 148 (23%) of 647 dogs and mild focal cecitis or colitis was reported in 25 (4%) of the 647 dogs (Hottendorf and Hirth, 1974). Parvovirus causes acute enteritis in weaned dogs but is largely controlled by vaccination. Granulomatous inflammation was reported in both large and small intestines. *Toxocara* granulomas occur in the wall of the intestine (Barron and Saunders, 1966). Granulomas were observed in the large intestine of 1%–3% and in the small intestine of 24% of young beagles (Glaister, 1986). Small intestine granulomas were reported in 6 (0.9%) of 647 dogs (Hottendorf and Hirth, 1974).

Mild hyperplasia of individual or aggregate lymph follicles in the lamina propria may be seen. Usually, these hyperplastic follicles correlate with an increased prominence of grossly observed nodules.

Intussusception was observed grossly in 1 (0.1%) of 1000 dogs (Hottendorf and Hirth, 1974).

Fritz et al. (1967) reported a congenital defect, Meckel's diverticulum, in the small intestine. Hernias in which intestines can protrude though congenital defects in the abdominal wall were observed grossly in 5 (0.5%) of 1000 dogs (Hottendorf and Hirth, 1974). Oghiso et al. (1982) report mucoepithelial cysts of the small intestine.

Rarely reported is the granular cell tumor of the cecum, characterized by large polygonal eosinophilic cells infiltrating into the lamina propria and submucosa (Sato et al., 2012).

Many metazoan and protozoan parasites have been reported in laboratory dogs, largely dependent upon the suppler. Nematodes are common and were observed in 23%–28% of young beagles (Glaister, 1986). Most were ascarids in the small intestines. Ascarids are the common large roundworms of dogs, *Toxocara canis* and *Toxocara leonina*. Usually in affected laboratory dogs, a few ascarids are observed in small numbers free in small intestine lumen. Ascarids were observed grossly in 165 (17%) of 1000 dogs (Hottendorf and Hirth, 1974) and reported in 2 (5%) of 37 untreated beagle dogs (Pick and Eubanks, 1965). Strongyloides (*Strongyloides stercoralis*) are small threadlike nematodes found in the small intestines, which were reported in 9 (1%) of 647 dogs (Hottendorf and Hirth, 1974). Whipworms, *Trichuris vulpis*, which occur in the cecum and colon, are uncommon in laboratory dogs. Trichuriasis was reported in 7 (19%) of 37 untreated beagle dogs (Pick and Eubanks, 1965). Hookworms (*Ancylostoma* spp.) usually affected young dogs and can result in pale oral membranes, anemia, and reduced growth rate. Heavy infections can result in black tarry feces. Ancylostomiasis was reported in 1 (3%) of 37 untreated beagle dogs (Pick and Eubanks, 1965). Tapeworms were observed grossly in 8 (0.8%) of 1000 dogs (Hottendorf and Hirth, 1974). The most common tapeworm is the "cucumber seed" *Dipylidium caninum*, which is usually asymptomatic but can result in diarrhea and anal pruritus. Giardiasis is important in young dogs. The disease can result in intermittent or chronic diarrhea, which may persist for several months accompanied by malabsorption of nutrients with reduced growth rate, weight loss, dull hair coat, and other clinical signs. It may be diagnosed clinically on the basis of cysts or trophozoites found in fecal samples. Generally, there is no evidence for histopathological diagnosis of the disease. Coccidiosis in young dogs can result in diarrhea and dehydration. Infections with *I. bigemina* can cause intestinal hemorrhage.

Liver

Gross and microscopic findings are very frequently observed in the liver. Andersen (1970) reported subcapsular cysts filled with serous fluid. Granulomatous inflammation has several forms. Granulomas were observed in 2%–4% of young beagles (Glaister, 1986) and in 23 (4%) of 647 dogs (Hottendorf and Hirth, 1974). Also, Maita et al. (1977) reported small granulomas in livers. The most common histological "lesions" in the liver of young beagles are small focal collections of histiocytes, lymphocytes, and an occasional neutrophil, at times accompanied by a few degenerate hepatocytes. The foci are commonly referred to as microgranulomas. Their pathogenesis and significance appear to be unknown. Microgranulomas were noted in 29 (91%) of 32 dogs (Yasuba et al., 1987). Granulomatous aggregations of mononuclear cells were reported by Oghiso et al. (1982). Granulomas can occur as a result of larvae of *T. canis* molting or dying during their migration through the liver on the way to mature in the intestine (Barron and Saunders, 1966). Eosinophilic phlebitis and periphlebitis suggestive of hypersensitivity reactions to migrating parasites were also present. Focal inflammation of veins (phlebitis) was very common and occurred in 409 (63%) of 647 dogs (Hottendorf and Hirth, 1974). Leukocyte foci are very common and occurred in 47%–60% of young beagles (Glaister, 1986).

Mild portal inflammation with and without bile duct hyperplasia was reported in 50 (8%) of 647 dogs (Hottendorf and Hirth, 1974). Inflammation of bile ducts (cholangitis) occurred in association with necrosis or other inflammatory changes and was not reported as a primary finding in control beagles. Bile duct hyperplasia was observed in 2 (0.3%) of 647 dogs (Hottendorf and Hirth, 1974).

Hepatocyte necrosis occurs in several forms and can be associated with several degenerative and inflammatory changes. Oghiso et al. (1982) reported the presence of necrosis, whereas Maita et al. (1977) reported necrosis at the base of hepatic ligaments. Focal necrosis and inflammation were very common and reported in 423 (65%) of 647 dogs (Hottendorf and Hirth, 1974). Focal subcapsular lipidosis and necrosis seen near the hilus of the liver at the attachment of hepatic ligaments is compatible with the so-called tension lesions seen in other species. These changes result from tension on the liver capsule and localized anoxia of adjacent hepatocytes. Hottendorf and Hirth (1974) report small, light-yellow foci in the caudate lobe near the porta hepatis. Rectangular or cubic acidophilic inclusions in the nuclei of hepatocytes (and renal tubular cells) have been frequently seen in the beagle and other dogs (as well as wolves, foxes, and jackals) (Thompson et al., 1959a,b; Richter et al., 1965). These structures are commonly referred to as acidophilic intranuclear inclusions (ACNs). They appear to be protein in nature. Their significance and origin remain obscure. They were described as cubic or rectangular intranuclear hyaline bodies by Maita et al. (1977), ACNs by Oghiso et al. (1982), and intranuclear rhomboid inclusions by Hottendorf and Hirth (1974). They were observed in 2 (0.3%) of 647 dogs (Hottendorf and Hirth, 1974). Hepatocytes of beagles also contain acidophilic, globular intracytoplasmic inclusions of equally obscure origin and significance (Murti and Borgmann, 1965; Holmes and Smith, 1969; Harleman et al., 1987). These structures are commonly referred to as periodic acid–Schiff (PAS)-positive, nonglycogenic intracytoplasmic inclusions. Studies indicate they consist of proteinaceous material and bound lipids. Andersen (1970) and Maita et al. (1977) reported these PAS-positive cytoplasmic inclusions. Small brown pigment granules may be seen in both hepatocytes and Kupffer cells. In the absence of biliary stasis, the pigment is usually lipofuscin or hemosiderin or both. Lipofuscin ("wear-and-tear" pigment) is PAS and oil red 0 positive. Hemosiderin is iron positive. Individual hepatocytes and Kupffer cells may contain both pigments. Oghiso et al. (1982) and Maita et al. (1977) reported lipofuscin deposition. Bile pigments may be present if biliary stasis is present.

Hepatocytic fatty changes also termed vacuolar degeneration, fatty degeneration, fatty metamorphosis, or lipidosis occur in young dogs. Oghiso et al. (1982) reported vacuolar degeneration and fatty degeneration. Focal fatty change was observed grossly in 78 (8%) of 1000 dogs (Hottendorf and Hirth, 1974). Focal subcapsular lipidosis and necrosis are associated tension lesions at the base of hepatic ligaments near the hilus of the liver.

Glucose is normally stored within hepatocytes as glycogen. This storage results in hepatocytic cytoplasmic vacuolation. Large amounts of glycogen are expected after a meal; however, surprising degrees of cytoplasmic vacuolation due to glycogen may be seen in dogs fasted overnight. Severe and widespread vacuolation of hepatocytes was observed in a young laboratory beagle with diabetes mellitus. Superficial necrolytic dermatitis was also present (Yoshida et al., 1996).

Gallbladder

Occasionally, cystic mucinous hyperplasia of the mucosal epithelium with copious mucin production can be seen, and rarely, hyperplasia of the mucosal epithelium lacking the cystic change or mucin production but with lymphocytic infiltration in the lamina propria is seen (Sato et al., 2012).

Hyperplasia of the lymphoid tissue with or without formation of lymphoid follicles is common. Fine brown or black crystalline gallstones were reported by Maita et al. (1977).

Pancreas

Ovoid, acidophic intracytoplasmic inclusions (often containing basophilic particles and surrounded by halos) may be seen in acinar cells of the pancreas. Hartman et al. (1975) have shown the inclusions to consist of whirls of rough endoplasmic reticulum, vacuoles, and cytoplasmic

organelles in various stages of decomposition and to be similar to dense ribosomal autophagic vacuoles. Apoptosis of the acinar cells is noted occasionally (Sato et al., 2012).

Oghiso et al. (1982) reported cytoplasmic alternative changes in acinar and islet cells, without specific descriptions of the changes. Edema and unspecified cellular infiltrations were also reported by Oghiso et al. (1982). Chronic focal pancreatitis was reported in 8 (1.2%) of 647 dogs (Hottendorf and Hirth, 1974). *Toxocara* granulomas were reported by Maita et al. (1977) and Barron and Saunders (1966).

Nesidioblastosis characterized by irregular proliferation of ductular cells between acinar cells, irregularly shaped islets, and small clusters of endocrine cells closely associated with ductules or intercalated ducts is seen rarely and is characterized as a proliferative lesion in the pancreas (Sato et al., 2012). The number and size of islets were markedly reduced in a young laboratory beagle with diabetes mellitus. Only glucagon-positive cells were demonstrated. The liver had severe hepatocytic vacuolation. Also a superficial necrolytic dermatitis was present (Yoshida et al., 1996).

Respiratory System

Anatomy and Histology

The respiratory system comprises the nasal cavity, paranasal sinuses, nasopharynx, larynx, trachea, and lungs. The principal function of the respiratory system is the exchange of gases (oxygen and carbon dioxide). Other functions include the warming, humidifying, and cleansing of incoming air; regulating airflow; olfaction; phonation; and temperature control of the whole organism (Dellman, 1976a). Important immunological and metabolic functions are also attributed to the respiratory system (Yates, 1988).

Nasal Cavity

The nasal cavity is the facial portion of the respiratory system and is also known as the internal nose (Miller et al., 1967). The part of the head known as the nose consists of an internal nose (a mucosa-lined cavity) and an external nose comprising bones (incisive, maxillae, nasal) and movable cartilages (nasal cartilages). The bones give rise to an elaborate system of bony scrolls (turbinates) that are covered by mucous membranes of the nasal cavity. The nasal cartilages surround the nostrils and nasal vestibule and direct the flow of air into the various passages (meatuses) among the scrolls. On inspiration, air enters the nasal cavity through paired nostrils (flares, singular: naris) and is drawn over four types of epithelium (stratified squamous, transitional, respiratory, olfactory) (Adams and Hotchkiss, 1983) before it leaves via the paired choanae (internal nares) to enter the nasopharynx. The rostral (anterior) 20%–35% of the nasal cavity is lined by thick stratified squamous epithelium. Transitional epithelium lines the next 15%–20%. Ciliated pseudostratified columnar (respiratory) epithelium lines the next 40%–50%, and olfactory epithelium lines the remainder of the cavity. The area lined by respiratory epithelium is the most vascular of the four regions. Nasal glands, scattered throughout the nasal mucosa, are present in greatest density in the olfactory region. Plasma cells are most abundant rostrally. Lymphocytes are present throughout the nasal mucosa but frequently occur as nodular masses in the caudal portion, forming grossly visible nodules near the choanae (Adams and Hotchkiss, 1983). The nasal and oral pharynges have been described with the digestive system.

Larynx

The laryngeal ostium is guarded by a cartilaginous valve (epiglottis) that prevents inspiration of food and water and controls the volume of air entering the lower respiratory system.

Trachea. The trachea is a flexible tubular connection between the larynx and lungs. Its flexibility is derived from a skeleton of C-shaped hyaline cartilages connected longitudinally by fibroelastic tissue and closed dorsally by smooth muscle and connective tissue (Miller et al., 1967). The tracheal mucosa is covered by respiratory epithelium in which the population of individual cell types (ciliated, preciliated, basal, secretory) varies significantly anteriorly and posteriorly and even dorsally and ventrally (Schwartz, 1987). Ciliated cells account for the bulk of the tracheal mucosal cells and are responsible for moving secretions. Glands of the tracheal mucosa are predominantly serous with occasional mucous acini (Dellman, 1976a).

Bronchi and Bronchioles

The trachea terminates by the formation of the right and left principal bronchi. The principal bronchi divide into lobar bronchi (secondary bronchi), which are named according to the lobe supplied. Lobar bronchi divide into segmental bronchi and the branching continues until respiratory bronchioles are formed. As bronchi penetrate into the lungs, they become embedded in a sheath of loose connective tissue, which contains lymphatics, nerves, bronchial vessels, and pulmonary arteries. Bronchi are kept patent by overlapping curved cartilages. When terminal bronchioles reach a diameter of 1 mm. or less, cartilages are no longer found (Miller et al., 1967). Bronchi bronchioles are lined by respiratory epithelium containing three general categories of secretory cells: serous, mucous, and nonciliated secretory bronchiolar (NBE or Clara) cells (Schwartz, 1987). Clara cells are present mainly in the peripheral airways. Clara cells have been shown to be the site of cytochrome P-450-dependent mixed function oxidase activity in the lung (Gill, 1982). Tubuloacinar mucous or mixed glands are located in the submucosa. Characteristically, glandular elements (and goblet cells) decrease as the bronchi decrease in size. In general, all bronchioles lack cartilage, the columnar epithelium gradually becomes cuboidal epithelium, and goblet cells are gradually replaced by Clara cells. Respiratory bronchioles give rise to alveolar ducts, alveolar sacs, and pulmonary alveoli. Alveoli are lined by three types of epithelium: type 1 alveolar epithelial cell (squamous pneumocyte), type 2 alveolar epithelial cell (secretory pneumocyte), and type 3 pneumocytes (brush cells) (Schwartz, 1987). Type 1 cells appear to line the alveolus and type 2 to produce pulmonary surfactant. The function of type 3 cells is unknown. Intra-alveolar macrophages are also important components of alveoli. There appear to be permanent lymphoid structures beneath the bronchial mucosa (localized infiltrations of the lamina propria with lymphocytes have long been recognized) that are comparable to those that constitute the GALT of the intestine. The lymphoid structures in the lung have been called bronchus-associated lymphoid tissue or BALT (Gill, 1982).

Lungs

The lungs (right and left) are divided into lobes. Each lung has an apical (cranial), cardiac (middle), and diaphragmatic (caudal) lobe. The right lung also has an intermediate (accessory) lobe. The pulmonary pleura is thin and adheres tightly to the surfaces of the lung and follows all its irregularities. The pleura is covered by a pavement of flat mesothelial cells and contains elastic, collagenous, and smooth muscle fibers. The subserosa of the pleura contains a superficial lymph vessel system that drains the pleura through the interlobular septa to the hilus of the lung. A deep lymph vessel system is oriented around the bronchial tree. It too drains toward the interlobular septa. Distended portions of the superficial lymphatic system may be prominent enough to be seen grossly, usually as clear, interconnecting, threadlike tubules. Pulmonary lymphatics drain into the right, middle, and left tracheobronchial lymph nodes. Smaller, bronchopulmonary lymph nodes are occasionally seen. When present, they lie on the surface of primary bronchi.

The lung (in common with the liver) has two blood supplies: bronchial arteries, which provide oxygen for the conducting airways, and pulmonary arteries, which deliver blood to the alveoli for

oxygen–carbon dioxide exchange. Bronchial arteries form plexuses in the bronchial wall. Pulmonary arteries branch more frequently than the airways and eventuality supply the most peripheral parts of the alveoli with blood via a capillary network. The origin and relationships of veins are not as well defined as the arteries.

Necropsy and Laboratory Techniques

Disease of the respiratory system produces a wide variety of clinical signs. Some of the signs may indicate pathology within a specific portion of the system, others are less specific. Pathology of the nasal cavity may be indicated by nasal discharge, snorting, sneezing, and nasal rubbing. Disease of the larynx and trachea may be indicated by dyspnea (difficulty or distress in breathing), stridor (high-pitched noisy respiration), gagging or retching (striving to vomit), and coughing. Coughing and dyspnea may also indicate problems in the lower respiratory tract, as does tachypnea (very rapid respiration) or hyperpnea (deep and rapid respiration). Rales (abnormal respiratory sounds heard on auscultation) may be detected. Many varieties of rales are described. Cyanosis (bluish discoloration of the skin and mucous membranes) may indicate problems in either the respiratory or cardiovascular system. Pulmonary cyanosis is the result of poor oxygenation of blood in the lungs. Many special procedures have been used in the morphological study of the respiratory system, including airway and vascular perfusion with a variety of fixatives, formalin vapor fixation of the lungs, whole lung sections (macrosections), thick and thin histological sections, vascular injection with colored latex, silicone rubber casts of airways, in vivo rapid freezing of the lung, stereology, and, of course, scanning and transmission electron microscopy (Dungworth et al., 1976). The complexities of the respiratory system dictate that a variety of techniques are required for adequate evaluation of toxic effects in inhalation studies. However, the basic necropsy and fixation procedures that are essential for inhalation studies are also recommended for general toxicology studies. The procedures add neither cost nor time to the postmortem examination. The critical step is perfusion of the lungs (or one or more lobes) with fixative either by the trachea or primary bronchus at a pressure that is adequate to inflate the lungs to approximately normal full expansion. After perfusion, the trachea (or bronchus) is closed (clamped or ligated) and the lung is fixed in the expanded state. Simply immersing pieces of collapsed lung in fixative is not acceptable except when the lungs are massively consolidated and edematous or contain large solid tumors (Dungworth et al., 1976). Even in the presence of such lesions, it is usually worthwhile to attempt to fix a portion of the lung by airway perfusion. Airway perfusion should be done at 30 cm of fluid (water) pressure. (Ideally, the lungs should be supported in a bath of fixative while being perfused.) Airway perfusion not only restores normal dimensions and configurations to the lung, it also provides a large volume of fixative in intimate contact with all surfaces of respiratory tree. The disadvantages of airway perfusion are the dislocation of exudates and distension of the tissue spaces around pulmonary vessels (the so-called edema artifact) (Dungworth et al., 1976). The edema artifact is dealt with during the microscopic interpretation. Immersion fixation of an additional affected portion of lung can compensate for the displacement of exudates by perfusion.

The lungs should be in view as the thoracic cavity is opened. The customary approach is to catch a glimpse of the distended lungs as the diaphragm is incised close to its sternal and costal attachments. Healthy lungs collapse as air enters the thorax. Failure of the lungs to collapse should be noted by the prosector. The thoracic cavity is opened preferably by cutting the ribs just dorsal to costochondral junctions to give adequate exposure of the thoracic contents. As noted earlier, all of the thoracic organs should be examined in situ and the tongue, larynx, trachea, lungs, heart, great vessels, and thymus removed in toto. The larynx and trachea are incised along their dorsal surface (with the prosector being alert to unusual content) to the bifurcation of the trachea. The lumina of the primary and lobar bronchi are inspected. The extent to which major airways need to be opened depends upon the type and amount of gross pathology. At least two lobes of the lung should be left

intact for intrabronchial perfusion. A compromise must be made between thoroughness and practicality in deciding upon the number of lobes to perfuse and the number of blocks to take from each lobe for microscopic examination. Perfusion of two lobes (one a cranial lobe, the other a caudal lobe) seems to be reasonable for general toxicology studies. After fixation, samples (blocks) should be taken from the dorsal (hilar) and ventral (peripheral) aspects of each lobe. The trachea is usually represented by a segment taken from the midsection. If the nasal cavity needs to be examined at necropsy, it may be split sagittally. If specimens are required, one or both halves of the nasal cavity may be fixed and tissue blocks removed after decalcification. Nasal passages should be gently flushed with fixative to remove air and any material that would prevent intimate contact between the nasal epithelium and the fixative. The frontal sinus should be opened as a routine matter when the calvarium is removed to expose the brain.

Pathology

Pathological findings reported for the respiratory system in untreated young dogs have been largely limited to the lungs. Erosion and/or ulceration of the laryngeal mucosa is noted rarely and focal inflammatory cell infiltration is noted occasionally (Sato et al., 2012). Chronic tracheitis was observed in 11 (2%) of 647 dogs on 39 studies (Hottendorf and Hirth, 1974). Hyperplasia of the tracheal mucosal epithelium and squamous metaplasia is seen frequently in the region near the bifurcation (Sato et al., 2012).

Inflammatory lesions of the lungs are the most frequent respiratory system findings. These lesions involve many locations and tissues resulting in numerous diagnoses being reported including perivasculitis, peribronchiolitis, endobronchiolitis, bronchitis/bronchiolitis, bronchopneumonia, pneumonitis, focal interstitial pneumonia, fibrosing alveolitis, fibrous thickening of alveolar walls, pleuritis, subpleural fibrosis, pulmonary helminthiasis, granulomatous pneumonitis, and various pulmonary granulomas (Pick and Eubanks, 1965; Hottendorf and Hirth, 1974; Oghiso et al., 1982; Glaister, 1986). Most of these pulmonary findings were thought to be due to infections by the lung worm, *Filaroides hirthi*. However, it should not be concluded that all pulmonary lesions are due to lungworm infection; some are believed to result from *T. canis* infections, foreign body emboli from intravenous injection sites, inhaled sawdust bedding, and infectious agents.

Pneumonia due to *F. hirthi* infection is probably the most common nontreatment-related respiratory finding seen in the laboratory beagle. The true incidence of the disease in laboratory dogs (and the canine population at large) is unknown. Clinical signs of infection are rare. The diagnosis can usually be made at necropsy based on the finding of tan, green, or gray subpleural nodules ranging from 1 to 5 mm (and larger) in diameter. A granulomatous response is evoked by dead or degenerating worms. Focal granulomatous interstitial pneumonitis is often seen when remnants of the parasite can no longer be identified. *F. hirthi* has a direct life cycle with infective first-stage larvae in the feces. Most pups are probably infected by their dams (Dungworth, 1985). Hirth and Hottendorf (1973) first reported the pathological changes associated with *F. hirthi* infection in the laboratory beagle. The parasite has been further characterized in subsequent articles (Georgi and Anderson, 1975; Georgi et al., 1975).

Granulomatous inflammation or granulomas were observed in 6%–7% of young beagles (Glaister, 1986) and in 75 (12%) of 647 dogs (Hottendorf and Hirth, 1974). Granulomatous nodules were also reported by Oghiso et al. (1982). Granulomatous pneumonitis was observed in 13 (35%) of 37 untreated beagles (Pick and Eubanks, 1965). Both lungworms and migrating ascarid larvae can cause granulomas in the lungs. Living nematodes were observed in the lungs from 2% to 6% of young beagles (Glaister, 1986). Granulomas can occur as a result of larvae of *T. canis* molting or dying during their migration through the lungs on the way to mature in the intestine. Barron and Saunders (1966) and Maita et al. (1977) reported *Toxocara* granulomas in the lungs. Vascular microgranulomas were attributed to emboli from intravenous injection sites in 8 (1%) of 647 dogs

(Hottendorf and Hirth, 1974). Cholesterinic granulomas were reported in 1 of 647 dogs (Hottendorf and Hirth, 1974). Leukocyte foci are very common and occurred in 17%–18% of young beagles (Glaister, 1986).

Bronchopneumonia is unusual in laboratory dogs because of vaccination programs against canine distemper. Canine distemper virus infections can cause viral pneumonias and predispose dogs to bacterial pneumonia. Bronchopneumonia was observed in 3 (8%) of 37 untreated beagles (Pick and Eubanks, 1965). Bronchopneumonia apparently due to *Mycoplasma* spp. was reported in four laboratory beagles (Kirchner et al., 1990). Localized bronchitis and bronchiolitis occurred in 2%–8% of young beagles (Glaister, 1986). Perivasculitis and peribronchiolitis were observed in 329 (51%) of 647 dogs (Hottendorf and Hirth, 1974). Subpleural fibrosis and endobronchiolitis were reported in 139 (21%) of the 647 dogs (Hottendorf and Hirth, 1974). Pneumonitis or interstitial pneumonia is common and occurred in 22%–28% of young beagles (Glaister, 1986). Focal interstitial pneumonia was observed in 111 (17%) of 647 dogs (Hottendorf and Hirth, 1974). Fibrosing alveolitis, a fibrous thickening of alveolar walls, was observed in 1%–3% of young beagles (Glaister, 1986). Pleuritis occurred in 2%–8% of young beagles (Glaister, 1986). Other findings in the lungs included atelectasis where areas of collapsed alveoli are present (Oghiso et al., 1982), as well as pulmonary hyperemia or congestion, and hemorrhage. Hyperemia or congestion occurs as an active component of inflammation or passive component of poor circulation from heart diseases and agonal events. Pulmonary hyperemia was observed in two (5%) and hemorrhage occurred in 1 (3%) of 37 untreated beagles (Pick and Eubanks, 1965). Edema may accompany hyperemia and congestion. As noted earlier, edema must be distinguished from the artifact that can result during perfusion of the lungs. Anthracosis, pigmentation of the lungs, was reported in 19 (51%) of 37 untreated beagles (Pick and Eubanks, 1965). This finding is unusual because most laboratory dogs are not exposed to air pollution or coal dust.

Cardiovascular System

Anatomy and Histology

The cardiovascular system is a closed system of tubes (blood vessels) containing a liquid tissue (blood) that is circulated by a four-chambered, double pump (heart). Blood is a complex tissue that serves several functions, including respiratory (transport of oxygen from the air in the lungs to the tissues and carbon dioxide in the tissues to the lungs), nutritive (conveys food materials from the digestive tract to the tissues), excretory (transports waste products from tissues to organs of excretion), homeostasis (maintains a dynamic equilibrium of water, pH, and electrolyte concentration), regulation of body temperature (functions in heat transport), chemical communication, and protection (circulates hormones and antibodies) (Cronkite, 1973).

Blood Vessels

The blood vessels comprise an enormous network of tubes (vascular system) that serve as a distributing system (arteries), a diffusion and filtration system (microcirculation), and a collecting system (veins).

Arteries. The first arteries in the distributing system are elastic arteries (aorta, pulmonary artery, brachiocephalic trunk, etc.), which merge with muscular arteries that conduct blood to various organs and regions of the body. Elastic arteries have a high content of elastic fibers and are characterized by large lumina and relatively thin walls. Most arteries in the body are muscular arteries, named because of their abundant smooth muscle content. Muscular arteries have smaller lumina and thicker walls than elastic arteries. In most muscular arteries, the wall thickness represents one-fourth of the vessel diameter (Simionescu and Simionescu, 1977).

Microvasculature. The arterial circulation connects with the venous circulation by way of the microvasculature. The microvasculature consists of the arterioles, capillaries, and venules. Arterioles are the smallest arteries in which the media is reduced to a layer of one or two smooth muscle cells. Arteriole lumina are small (less than 300 µm), but the arteriole wall is relatively thick (about one-half the diameter of the vessel).

Capillaries. The term "capillary" is restricted to minute vessels that consist only of endothelium, basal lamina, and a few pericytes (Simionescu and Simionescu, 1977). The inner diameter of blood capillaries ranges from 5 to 10 µm. Three principal types of blood capillaries have been described: continuous capillaries, fenestrated capillaries, and discontinuous capillaries (sinusoids). Continuous capillaries are characterized by a continuous endothelium and found in skeletal, cardiac, and smooth muscle; connective tissue; central nervous system; exocrine pancreas; and gonads. Fenestrated capillaries have transcapillary openings (fenestrae) approximately 600–800 A in diameter. Fenestrated capillaries are found in the mucosa of the gastrointestinal tract, endocrine glands, renal glomerular and peritubular capillaries, choroid plexus, and ciliary body.

Discontinuous capillaries are thin-walled vessels with large gaps (up to thousands of angstroms in diameter) in the endothelium and basal lamina. Discontinuous capillaries are found in the liver, spleen, and bone marrow.

Venules. These are similar to capillaries but are larger. The immediate postcapillary venules are characterized by the presence of pericytes (pericytic venules). Pericytic venules are drained by venules of increasing diameter whose media contain one or two thin layers of smooth muscle cells (muscular venules) (Simionescu and Simionescu, 1977).

Veins. From venules, the blood is collected in veins increasing in size, eventually becoming large veins such as the venae cavae and pulmonary vein. Blood vessels have three basic tunics: intima, media, and adventitia. The intima comprises (at most) the endothelium, basal lamina, subendothelial connective tissue, and the internal elastic lamina. The media is composed of muscular cells, elastic lamellae, and the external elastic lamina. The adventitia contains connective tissue and fine collagen and elastic fibers. As indicated earlier, the proportions and composition of the tunics vary with types of blood vessels.

Heart

The heart is basically a three-tunic segment of the vascular system with the middle layer (media) greatly developed. The outer tunic (adventitia) is called the epicardium. The media is termed the myocardium and the inner tunic (intima) the endocardium. The myocardium forms most of the mass of the heart. The heart is formed of four chambers: two atria and two ventricles.

Atria. The atria are the thin-walled, low-pressure chambers of the heart. The right atrium receives blood from the systemic circulation and contains openings for the coronary vein, posterior vena cava, anterior vena cava, and the azygos vein. The right atrium opens into the right ventricle through the right AV orifice. The left atrium receives blood from the pulmonary circulation and contains several openings for the pulmonary veins. The left atrium opens into the left ventricle through the left AV orifice. The atria are sometimes incorrectly referred to as the auricles. The auricle (L. little ear) is the blind-ending, forward-directed outpouching from each atrium.

Atrioventricular valves. The AV valves are intake valves to the ventricles. The right AV valve (known as the tricuspid valve in humans) consists basically of two cusps in the dog (Miller et al., 1967). The cusp adjacent to the interventricular septum is the septal, or dorsal, cusp. The cusp adjacent to the outer wall is the lateral, or ventral, cusp. The left AV valve (mitral valve in humans) is basically bicuspid in the dog also, but the subdivisions are indistinct. The cusp adjacent to the interventricular septum is the dorsal cusp. The cusp adjacent to the ventricular wall is the ventral cusp.

Ventricles. The right ventricle pumps systemic blood to the lungs by way of the pulmonary orifice. The left ventricle pumps oxygenated blood to the systemic circulation by way of the aortic

orifice. The valves guarding the pulmonary and aortic orifices are similar, with each consisting of three semilunar cusps.

Both ventricles contain muscular projections known as papillary muscles. Papillary muscles are larger in the left ventricle. Thin fibromuscular cords (chordae tendinae) arise from the apices of the papillary muscles and attach to the AV valves. The chordae tendinae keep the AV valves from being pushed into the atria during contraction of the ventricles. The ventral portion of the thick wall of the left ventricle forms the apex of the heart. The base of the heart is the dorsal portion where atria and ventricles join and valves are inserted into narrow fibrous rings.

Functionally, there are two general types of myocardial cells: muscle cells and impulse formation and conduction cells. The cardiac muscle cells have been described with the musculoskeletal system. The impulse-formation and conduction cells form the SA node, AV node, and AV bundle.

Sinoatrial node. In the dog, the SA node is located in the right atrium in the terminal crest at the confluence of the anterior vena cava, sinus venarum cavarum, and auricular orifice (Miller et al., 1967).

Atrioventricular node. This node is also located in the right atrium, about 5 mm cranioventral to the opening of the coronary sinus and craniodorsal to the septal cusp of the right AV valve.

Atrioventricular bundle. This bundle runs forward and downward from the AV node. The bundle divides into right and left branches, which lie closely under the endocardium of the septal wall of the right and left ventricles. In the dog, the Purkinje fibers of AV bundle are readily identified by their large diameter, centrally located large spherical nucleus, and scarce myofibrils (Dellman and Venable, 1976).

Pericardium. The heart is enclosed in a fibroserous envelope (pericardium, heart sac). The pericardium is divided into an outer fibrous part and an inner serous part. The serous pericardium in turn is divided into two parts or layers. The visceral layer is the epicardium and is attached firmly to the heart except in the region of coronary grooves. The parietal layer is fused with the fibrous pericardium. The pericardial cavity is located between the two layers of the serous pericardium and typically contains a small quantity of clear, light yellow fluid (pericardial fluid). The fibrous pericardium forms the strong outer part of the heart sac.

Lymphatic circulatory system. There is also a lymphatic circulatory system that is made up of a network of channels, which originate in connective tissue spaces as anastomosing capillaries. Although called a circulatory system, the lymph flows in just one direction, toward the thorax. In the dog, the thoracic duct is the chief return channel for lymph. The thoracic duct generally joins the venous circulation near the junction of the left jugular vein with the anterior vena cava (Miller et al., 1967).

Necropsy and Laboratory Techniques

Clinical signs of cardiovascular disease include weakness, fatigue, syncope (fainting), reluctance to lie, cyanosis, ascites, subcutaneous edema, venous distension, dyspnea, polydipsia, abnormal pulse, and a wide range of abnormal auscultatory sounds.

The initial examination of the heart and major vessels (pulmonary trunk, aorta, brachiocephalic trunk, left subclavian artery, and anterior and posterior venae cavae) is made with the thoracic organs in place. Abnormal location, size, shape, and proportion are looked for. The initial examination of the pericardial sac is also done in situ. The sac is grasped near the apex of the heart and a small incision made to access the volume and character of the pericardial fluid. If indicated, a sample of the contents may be taken at this time. The sac is then fully opened and both of its surfaces examined. The thoracic organs are removed. The aorta and posterior venae cavae are severed at the diaphragm. The remaining major vessels (or branches of them) are severed at the chest wall or thoracic inlet. In separating the heart from the lungs, the pulmonary veins are cut at the left atrium and the pulmonary trunk left attached to the heart. The pericardial sac is removed.

A routine should be followed in opening the heart. One that is easily mastered is to follow the flow of blood through the heart, beginning with the right atrium. The posterior vena cava is opened.

The incision is continued into the wall of the right atrium just dorsal and parallel to the coronary groove and into the right auricle. The anterior vena cava is not incised to avoid disturbing the SA node. The right ventricle is opened by cutting along the junction of the right ventricular free wall and ventricular septum. The incision begins caudally at the right AV orifice and proceeds to the apex, conus arteriosus, and through the pulmonary orifice and pulmonary trunk. Most papillary muscles of the right ventricle arise from the apical portion of the interventricular septum. With care, the incision separating the wall from the septum will leave the papillary muscles attached to the septum. The left atrium is opened through one of the pulmonary veins. The free atrial wall is cut just dorsal and parallel to the coronary groove with the cut continuing into the left auricle. An incision is then made caudally through the left AV orifice continuing between the posterior papillary muscle of the left ventricle and the ventricular septum. The incision is extended to the apex and into the anterior wall. Scissors are then inserted through the aortic outflow tract and an incision is made through the aortic orifice, wall of the aortic arch, and the aorta.

The brachiocephalic trunk and left subclavian arteries should also be opened. All chambers, surfaces, and valves of the heart are now exposed for detailed examination. It is suggested that the gross examination also be systematic, following the flow of blood through the heart. The heart is usually weighed in toxicology studies. Heart weight is essentially a measure of the mass of the myocardium; therefore, as much of the nonmyocardial tissue as possible should be removed. The pericardial sac has been removed. The pulmonary trunk and aortic arch are severed just distal to their respective valves. Any great length of anterior or posterior vena cava should be removed. Fat within the coronary grooves accounts for a small fraction of heart weight and need not be removed; and, of course, the heart valves are left intact. If extracardiac tissues are removed in a consistent manner, the resulting heart weights should be representative. If the heart is opened in the manner described, it is easily subdivided into various components: right ventricular wall, left ventricular wall, interventricular septum, atrial walls, and interatrial septum.

Weighing individual components was useful in documenting the cardiac hypertrophy of cor pulmonale in beagle pups (Brewster et al., 1983).

The prosector should be alert to lesions in the larger systemic vessels during the course of the postmortem examination. The abdominal aorta and external iliac entries should be examined in situ after the abdominal and pelvic viscera are removed. Selection of tissues for histological study should be as standardized as the gross examination. A complete histological examination would include samples of the conducting system; each valvular region, walls, and septa of the atria and ventricles; and segments from the pulmonary trunk and thoracic and abdominal aortae. The entire heart of the average beagle may be fixed after the right and left ventricular walls are removed and subdivided into two or three segments. The atrial and ventricular septa should be subdivided by vertical cuts that leave the SA and AV nodes intact.

Pathology

A variety of gross and histological changes have been reported for the cardiovascular system of laboratory beagles.

Major blood vessels, arteries, and veins. Collection of blood samples from the cephalic vein rarely causes sufficient injury to result in diagnosed lesions. Spontaneous gross lesions included congenital patent ductus arteriosus in 1 (0.1%) and pulmonic stenosis in 1 (0.1%) of 1000 dogs on 39 studies (Hottendorf and Hirth, 1974). Inflammation of arteries, arteritis, occurred in 2% of young beagles (Glaister, 1986). Idiopathic canine polyarteritis also termed panarteritis or periarteritis is a spontaneous arterial disease that occurs in both immature and mature beagles and mongrels. The disease occurs in acute, subacute, and chronic forms and can be a complicating factor

in toxicology studies. Incidences of 1.3% in 331 dogs to 3.3% in 668 dogs have been reported with predominance in the right coronary and epididymal arteries (Kerns et al., 2001). Extramural periarteritis affecting the coronary artery was reported in 1 (0.1%) of 647 dogs (Hottendorf and Hirth, 1974). Arteritis and periarteritis were reported in 10 of the 59 dogs with detrusor myopathy (Cain et al., 2000). Calcification of the round ligament of the bladder (remnant of the umbilical artery of the fetus) was seen, but not listed as a significant finding (Hottendorf and Hirth, 1974). Focal medial degeneration of the aorta was observed in 1 (0.1%) of 647 dogs (Hottendorf and Hirth, 1974). Mineralization in the aortic media near the base of the heart occurred in 2%–3% of young beagles (Glaister, 1986). Vascular microgranulomas in the lungs were attributed to emboli from intravenous injection sites in 8 (1%) of 647 dogs (Hottendorf and Hirth, 1974). Hypertrophy of muscular arteries must be interpreted with caution, because they may undergo postmortem contraction and appear to be unusually thick walled. The contracted arteries are usually devoid of blood and their internal elastic lamina has a scalloped appearance in cross section. Postmortem imbibition of hemoglobin can produce a diffuse red staining of the intima in both the heart and blood vessels that simulates hemorrhage.

Heart valvular endocardiosis is probably the most frequently observed incidental finding in the heart. Endocardiosis chiefly affects the septal cusp of the right AV valve in young beagles. The cusp appears to be diffusely or irregularly swollen, soft, and glistening. Valvular telangiectasis or angiectasis, also termed hematocyst or hematoma, appears to be a congenital malformation (Hubben et al., 1963). The lesion usually appears as a dark red cyst, 1–5 mm in diameter that protrudes from the atrial surface of the cusp. The septal cusp of the right AV valve is also the most common site for valvular telangiectasis. Valvular telangiectasis was observed grossly in 4 (0.4%) of 1000 dogs (Hottendorf and Hirth, 1974).

Neither these cysts nor the valvular endocardiosis appear to be of any functional significance. Inflammation of the myocardium, myocarditis, is infrequent. Parvovirus causes myocarditis in puppies and recently weaned dogs but has been largely controlled by vaccination. Focal myocarditis was observed in 4 (0.6%) of 647 dogs (Hottendorf and Hirth, 1974). Fibrosis is a component of chronic inflammation and has been reported throughout the heart. Focal myocardial fibrosis and calcification or mineralization were observed in 2 (0.3%) of 647 dogs (Hottendorf and Hirth, 1974). Chronic valvular fibrosis observed grossly in 27 (3%) of 1000 dogs (Hottendorf and Hirth, 1974). Toxocara granulomas have also been reported in the myocardium (Barron and Saunders, 1966). Granulomas occurred in 2 (0.3%) of 647 dogs (Hottendorf and Hirth, 1974). Leukocyte foci were reported in myocardium of 1% of young beagles (Glaister, 1986). Fatty infiltration of the myocardium was observed in 13 (35%) of 37 untreated beagles (Pick and Eubanks, 1965). Sato et al. (2012) noted that proliferation of the epicardial mesothelium, considered to be secondary to physical irritation, can be occasionally seen in the right or left atrial epicardium. Focal mesothelial proliferation was noted in 1 (3.1%) of 32 dogs (Yasuba et al. 1987).

Apparent degenerative changes within the myocardium must be interpreted with caution for, as with skeletal muscle, many artifacts may result from handling and from contraction of myofibrils during fixation. Postmortem imbibition of hemoglobin produces a diffuse red staining of the intima of both the heart and blood vessels that simulates hemorrhage.

Chronic focal pericarditis was observed in 1 (0.1%) of 647 dogs (Hottendorf and Hirth, 1974).

Hemopoietic System

Anatomy and Histology

The hemopoietic system consists of the blood, bone marrow, and lymphoreticular tissues. Blood is a liquid tissue comprised of a fluid portion (plasma), red cells (erythron), white cells (leukon), and platelets (thrombon) (Payne et al., 1976).

Erythron

The erythron consists of the erythrocytes and their precursors. The primary function of the erythrocyte is to mediate the exchange of oxygen and carbon dioxide. In the adult dog, red cell production (erythropoiesis) occurs predominantly in the bone marrow; however, other organs (spleen, liver, lymph nodes, adrenals, kidneys) retain the potential to produce erythrocytes (extramedullary hematopoiesis) in adult life (Valli, 1985). The average erythrocyte life span for the dog is 110 days (Duncan and Prasse, 1986).

Leukon

The leukon consists of granulocytes (neutrophils, eosinophils, basophils), monocytes, and lymphocytes. In the adult dog, virtually all granulocytes are produced in the bone marrow.

Granulocytes

Neutrophils. Neutrophils are the most abundant of the circulating white blood cells (60%–77% in the dog) (Duncan and Prasse, 1986). After a brief period of circulation, neutrophils migrate to the tissues and remain there. Blood neutrophils are replaced about 2.5 times a day. The major function of neutrophils is to phagocytize and kill microbes.

Eosinophils. Like neutrophils, eosinophils are mobile phagocytes with antimicrobial properties, but they are not protective against bacterial infection. Of the circulating white blood cells, 2%–10% are eosinophils. Eosinophils are more numerous in tissues than in blood.

Basophils. These are weakly motile phagocytes. Basophils and mast cells (related functionally but not by origin) contain mediators of inflammation, including histamine, heparin, eosinophil chemotactic factor of anaphylaxis, platelet-activating factor, and other substances (Prasse, 1983). Basophils are rarely seen in the circulating blood.

Monocytes. Produced in the bone marrow, monocytes migrate into the blood and quickly enter into tissues (lung, liver, spleen, bone marrow, pleura, peritoneum, lymph nodes) to become tissue macrophages (Valli, 1985). They are the Kupffer cells of the liver and the osteoclasts of the bone. Monocytes represent 3%–10% of circulating white blood cells. The activities of the monocyte–macrophage system include phagocytosis, myeloproliferative control, antigen processing, and production of various cytokines.

Lymphocytes. In early embryonic life, lymphoid precursors from the yolk sac colonize the bone marrow and thymus (primary lymphopoietic organs). Progeny from the primary lymphopoietic organs in turn populate lymph nodes, spleen, tonsils, gut, and lung (secondary lymphopoietic organs). The bone marrow is the larger and more active primary lymphopoietic organ. The fate of most bone marrow lymphocytes is unknown (Wintrobe et al., 1981), but some enter the circulation and migrate to the secondary lymphopoietic organs to become the source of antibody-producing cells (B lymphocytes, bone marrow–derived cells). Most thymic lymphocytes experience an intrathymic death, but some enter the blood and seed out in the thymus-dependent areas of secondary lymphopoietic organs, where they become progenitors of cells involved in cell-mediated immunity (T lymphocytes, thymus-derived cells). Compared to other leukocytes, lymphocytes are long lived (weeks to years). They also are unique because they recirculate (Duncan and Prasse, 1986). The majority of permanently circulating lymphocytes are T cells. The majority of B cells remain in the lymphoid tissue. Lymphocytes comprise 12%–30% of the circulating white blood cells.

Thrombon. The thrombon consists of the circulating blood platelets and the megakaryocytes and megakaryoblasts of the bone marrow (Payne et al., 1976).

Blood platelets. Extramedullary megakaryopoiesis does occur and is most common in the lung but is also seen in the spleen, liver, kidney, and heart (Green, 1983). The normal blood platelet count in the dog is $2–9 \times 10^5$/mcl (Duncan and Prasse, 1986). The platelet number can be affected by splenic contraction (increases number of platelets) and congestion (decreases number). The circulating life span of platelets is approximately 10 days. The platelets' central role is in hemostasis. The major platelet reactions are adhesion to damaged endothelium, release of biochemical substances, causing vasoconstriction, and further platelet aggregation (Green, 1983).

Bone marrow. Bone marrow maintains an embryonic function throughout adult life. Bone marrow may be red (due to the hemoglobin contact of erythrocytes and their precursors) or yellow (due to fat cells). The bone marrow has two compartments: vascular and hematopoietic. The vascular compartment is supplied by nutrient arteries that divide and gradually narrow to capillaries and then open to large sinuses lined by discontinuous endothelium. The marrow lying between the sinuses is the hematopoietic compartment. The hematopoietic compartment of red marrow contains mostly hematopoietic cells and a few fat cells. Fat cells predominate in the hematopoietic compartment in yellow marrow.

Lymphoreticular Tissues

Lymphoreticular tissues include the thymus, spleen, lymph nodes, and lymphoid tissues of the digestive and respiratory systems.

Thymus

This is a light gray, multilobulated organ that lies in the cranial ventral part of the thoracic cavity almost entirely covered by the precardial mediastinum. At birth, the thymus is large and continues to grow during the first 3 months of postnatal life in the beagle (Andersen and Goldman, 1970). During the period from 6 to 23 months, the thymus undergoes progressive involution (Ploemen et al., 2003) but never completely disappears (Miller et al., 1967).

The thymus is a composite of epithelial and lymphoid tissues. Each thymic lobule is composed of a cortex that contains many lymphocytes (or cells that give rise to lymphocytes) and a medulla that contains epithelial and myoid cells (Valli, 1985). The epithelial cells of the thymus provide a unique environment essential for T-lymphocyte development. Various subsets of T cells are formed that are released and undergo final maturation in the spleen or other secondary lymphoid organs. The medullary epithelial cells form morphologically distinct clusters of cells (thymic corpuscles, Hassall's corpuscles) whose function is unknown. Examination of thymus from 120 healthy control beagles ages 6–23 months demonstrated medullary lymphoid follicles with occasional germinal centers in 70% of the dogs. The CD79 alpha marker confirmed that 97% of these dogs had B-cell-rich medullary areas. During involution, the B-cell areas and lymphoid follicles became less distinct (Ploemen et al., 2003).

Spleen

The spleens of domestic animals vary in their blood storage capacity and the relative amount of smooth muscle found in the capsule and trabeculae. The canine spleen has abundant venous sinuses capable of storing large amounts of blood and abundant smooth muscle in the capsule capable of considerable contraction (Brown and Dellmann, 1976). Splenic parenchyma is called pulp, most of which is red (red pulp) due to the presence of blood. The red pulp is almost entirely made up of splenic sinuses and thin plates of cells (splenic cords) lying between the sinuses.

Scattered throughout the red pulp are lymphoid nodules (splenic nodules) and lymphoid periarterial sheaths. The lymphoid nodules and sheaths constitute the white pulp. The lymphoid nodules represent concentrations of B lymphocytes; the sheaths are predominantly T lymphocytes.

The spleen has no afferent lymph supply; consequently, all antigens reach the spleen with the blood. The spleen filters unwanted elements from the blood (aged and damaged erythrocytes, particulate matter) and is a major secondary lymphoreticular organ, a secondary source of hematopoiesis, and a reserve pool of erythrocytes and platelets (Contran et al., 1989).

Lymph Nodes

Lymph nodes are the most organized of the lymphatic organs. External lymph nodes are found in the protective environment of small fat pads. Internal nodes are found in the mediastinum, mesentery, near the angles of many larger vessels, and the hilus of organs. Lymph nodes are usually named according to their location. The capsules of lymph nodes are perforated at various points by afferent lymphatics that empty into subcapsular sinuses. Branches of the sinuses (medullary sinuses) extend into the node and terminate at the hilus, where the efferent lymphatics emerge. Lymph percolates into and out of the parenchyma through gaps in the sinus walls. The cortex is made up of lymphoid nodules (primary nodules) whose size and morphology vary greatly. The primary follicles represent B-cell areas. Upon antigenic stimulation, the primary follicles enlarge and develop pale-staining germinal centers. B-cell progeny leave the germinal centers to either migrate to medullary cords where they become plasma cells or enter the blood lymphocyte pool (Valli, 1985). The paracortex (outer cortical area not occupied by germinal centers) is largely occupied by T cells. Medullary cords are papillary-like extensions of the paracortical cells toward the hilus of the node.

Necropsy and Laboratory Techniques

Anemia is defined as an absolute decrease in the packed cell volume, hemoglobin concentration, and red blood cell count (Duncan and Prasse, 1986). The clinical signs of anemia include pale mucous membranes, weakness, loss of stamina, dyspnea on exertion, tachycardia, and heart murmur. Icterus, hemoglobinuria, hemorrhage, and fever may also be seen depending upon the pathogenesis of the anemia. Enlarged superficial lymph nodes may indicate disease in the region being drained: parotid, mandibular, medial retropharyngeal lymph nodes (head and neck); superficial cervical lymph nodes (head, neck, thoracic limb, thoracic wall); axillary nodes (thoracic limb, thoracic wall, cranial and middle mammary glands); superficial inguinal nodes (abdominal wall, caudal mammary glands, penis, scrotum, entire pelvic limb); and popliteal (distal pelvic limb) (Miller et al., 1967). Bone marrow specimens (for histology and cytology) should be obtained as soon after death as possible (second in priority only to the eyes). Specimens may be easily gotten from the sternum.

For histology, one or two sternebrae or the distal femur may be cut longitudinally with a stout sharp knife. Segments are placed in fixative. One segment may be reserved for archival storage and the other decalcified and processed for paraffin embedding. Smears for marrow cytology and cell counting may be gotten from another sternebra. After removal of muscle, fat, and an intersternabral cartilage, pressure is applied by a sturdy pair of pliers to force marrow to the cut surface, where it is removed by means of a tapered artists brush (moistened in fetal bovine calf serum) and applied in streaks upon a glass slide. The specimen is air-dried. The prosector should be alert to abnormalities in individual lymph nodes during the course of the general postmortem examination. Specimens of both external and internal lymph nodes are usually taken for microscopic examination. Commonly, these are a medial retropharyngeal lymph node (the largest node in the head and neck, readily identified and easily removed) and a mesenteric lymph node. The anterior pole of the medial retropharyngeal lymph node may be discolored (tattoo ink from the ear). Mesenteric lymph nodes are often reddened (congested). Lymph nodes should be handled carefully to minimize artifacts. Searcy (1988) recommends that lymph nodes be fixed for about 1 hr prior to slicing (lymph nodes cut in the fresh state bulge through the capsule). Touch imprints may be prepared from the cut

surface of one pole before the node is placed in fixative. The size of the thymus varies considerably in short-term toxicology studies. The thymic involution begun prior to sexual maturity (Andersen and Goldman, 1970) and may be enhanced by the direct or indirect effect (malnutrition) of test compounds. Congestion of the spleen is common at necropsy, and it especially follows euthanasia with barbiturates. Several parallel slices should be made through the spleen to examine the pulp. One or two narrow slices should be placed in fixative.

Pathology

Of incidental changes that have been reported for the hemopoietic system of laboratory beagles, most involved the spleen.

Thymus

The size of the thymus varies greatly in dogs on toxicology studies depending upon the age and individual animal. Involution begins prior to sexual maturity. Atrophy or reduction in the size is an uncommon finding in untreated beagles and must be distinguished from involution. Proliferation of the thymic epithelium can be present, which may be associated with thymic involution (Sato et al., 2012). Lymphoid follicle formation can be noted in the medulla as an incidental change (Sato et al., 2012). Ectopic thyroid tissue was observed in the thymus of 1% of young beagles (Glaister, 1986). Also, thymic cysts were reported in 1% young beagles (Glaister, 1986).

Spleen

Many vascular changes occur in the spleen of laboratory dogs. Angiectasis or telangiectasis is a localized dilatation of blood vessels that are usually observed as dark blebs around the margins of the spleen. They can arise as blood-filled sinusoids that fail to empty during the splenic contractions associated with exsanguinations. Telangiectasis was observed in 17%–18% of young beagles (Glaister, 1986). Congestion or hyperemia is diffuse dilatation of blood vessels of the spleen. Congestion can be active as when associated with inflammation or passive when associated with poor blood circulation as with heart failure. Splenic hyperemia was observed in 1 (3%) of 37 untreated beagles (Pick and Eubanks, 1965). Congestion must be distinguished from hemorrhage. Hemorrhage is common in the spleen because of the large volume of blood contained in the spleen and the abdominal location. Splenic hemorrhage was reported in 1 (3%) of 37 untreated beagles (Pick and Eubanks, 1965). Hematomas are large localized areas of hemorrhage and infrequent. Hematomas were observed grossly in 2 (0.2%) of 1000 dogs (Hottendorf and Hirth, 1974). Hemosiderosis is the tissue deposition of iron pigment associated with the breakdown of red blood cells and can occur abnormally as a result of increased fragility of erythrocytes or following hemorrhage. Because of normal destruction of erythrocytes at the end their life span, the normal spleen has some degree of hemosiderosis. In dogs, hemosiderin is commonly associated with siderofibrotic nodules. Splenic hemosiderosis was reported in 17 (46%) of 37 untreated beagles (Pick and Eubanks, 1965).

Although the spleen is an active site for hematopoiesis in puppies, the production of blood cells by the splenic pulp, especially the erythroid series, is uncommon in adult dogs. Splenic extramedullary hematopoiesis was reported in 3 (8%) of 37 untreated beagles (Pick and Eubanks, 1965).

Small Gandy–Gamna-like bodies in the splenic capsule were reported by Maita et al. (1977). Gandy–Gamna bodies (an eponym from medical pathology) refer to firm, nodular discolorations seen on or within the spleen. In veterinary pathology, the lesions are commonly referred to as siderofibrotic nodules (Ishmael and Howell, 1967). In the dog, they may appear as small, irregular slightly raised nodules on the capsular surface to extensive irregular encrustations covering

large areas of the capsule and as nodules within the pulp. The color of the nodules varies from yellow to grayish brown. The nodules are seen most frequently on the margins of the spleen with concentrations at the extremities and also on the visceral surface with concentrations at the attachment of the gastrosplenic omentum. Microscopically, the nodules are fibrotic foci that are commonly calcified and contain brown pigment (hemosiderin) and bright yellow pigment (hematoidin, bilirubin) that are probably the end result of hemorrhage or marginal telangiectasis, as noted by Glaister (1986). They were observed grossly in 90 (9%) of 1000 dogs (Hottendorf and Hirth, 1974).

Few lymphoid tissue changes were reported. Oghiso et al. (1982) reported lymphoid hyperplasia and reticulosis in the spleen. Reticulosis is a form of lymphoid hyperplasia consisting predominantly of reticuloendothelial or histiocytic cells. Other than those associated with siderofibrotic nodules, most inflammatory changes were limited to granulomas. Maita et al. (1977) reported Toxocara granulomas in the spleen. Splenic fibrosis was observed in 1 (3%) of 37 untreated beagles (Pick and Eubanks, 1965). One or more masses of splenic tissue may be found in the gastrosplenic omentum in addition to the spleen itself. These are known as accessory spleens. They may be congenital, but apparently, many are acquired through traumatic rupture of the spleen (Valli, 1985). Accessory spleens were observed grossly in 1 (0.1%) of 1000 dogs (Hottendorf and Hirth, 1974).

Fritz et al. (1966) reported lymphosarcomas in the spleen and lymph nodes of beagles under 1 year of age.

Lymph Nodes

The lymph nodes most frequently processed and examined microscopically are mandibular, mesenteric, and medial retropharyngeal. Congestion of the mesenteric lymph nodes is commonly observed. Congestion can be physiological and normal when associated with digestion and active when associated with inflammation or passive when associated with poor blood circulation as with heart failure. Congestion must be distinguished from hemorrhage. Hottendorf and Hirth (1974) observed, but did not report as a lesion, congestion of the mesenteric lymph nodes.

Granulomatous inflammation was the most commonly observed inflammatory change in lymph nodes. These lesions, granulomas, were most frequent in the mesenteric lymph nodes and usually occurred as the result of Toxocara larvae migration (Barron and Saunders, 1966; Maita et al., 1977). Granulomas were reported in 21%–27% of young beagles (Glaister, 1986) and in 16 (2%) of 647 dogs from 39 studies (Hottendorf and Hirth, 1974). Inflammation or lymphadenitis can occur with generalized involvement of lymph nodes after infection with *Brucella canis* (Jubb et al., 1992). Lymphoid cell proliferation was observed in the lymph nodes of untreated dogs. Lymphoid hyperplasia in which there was a nonneoplastic increase in lymphoid cells, and plasmacytosis in which there was a nonneoplastic increase in plasma cells, was reported (Oghiso et al.; 1982). Lymphoid hyperplasia is often associated with inflammation and infection in adjacent tissues. It must be distinguished from early neoplastic changes. Fritz et al. (1966) reported lymphosarcomas, in the spleen and lymph nodes of beagles under 1 year of age. Sato et al. (2012) noted that brown pigment–laden macrophages can be seen accumulating predominantly in the medullary regions of the mandibular lymph nodes. Similarly, tattoo pigments also occasionally migrate to the mandibular lymph nodes (Sato et al., 2012). Accumulation of foamy macrophages can be seen occasionally in the lymphatic sinuses, especially in the mesenteric lymph nodes (Sato et al., 2012).

Other Lymphoid Tissues

Hottendorf and Hirth (1974) observed, but did not report as a lesion, mild hyperplasia of lymph follicles in the pylorus, small intestine, cecum, and gallbladder.

Endocrine System

Anatomy and Histology

Two systems have major coordinating responsibilities in the body. One is the nervous system and the other is the endocrine system. Glands of the endocrine system communicate principally by way of the blood through which raw materials and special releasing and inhibiting factors (hormones) are received and into which the glands' own secretions (hormones) are discharged. Glands that are universally recognized as endocrine glands are the hypophysis (pituitary gland), thyroid, parathyroids, adrenals, pancreas (pancreatic islets, endocrine pancreas), testes, and ovaries. The endocrine functions of other organs such as the pineal body, thymus, kidney, and gastrointestinal tract are beyond the scope of this chapter.

Hypophysis

The hypophysis is structurally and functionally a part of a complex hypothalamohypophyseal system (Dellman, 1976b). Traditionally, only two divisions of this system (adenohypophysis and neurohypophysis) are included in a discussion of the hypophysis. The adenohypophysis consists of the pars tuberalis, pars intermedia (intermediate lobe), and pars distalis (anterior lobe). The neurohypophysis consists of the pars nervosa (posterior lobe) and the infundibulum. The hypophysis has a dual embryological origin. The adenohypophysis arises as an upward evagination of the oropharyngeal ectoderm (the lining of the future oral cavity, Rathke's pouch). The neurohypophysis is a downward evagination of the brain (diencephalon).

The hypophysis of the dog is a slightly flattened ovoid body lying in a shallow concavity of the basisphenoid bone and attached to the base of the brain by a short hollow stalk (infundibulum). The hypophysis is partly surrounded by bone (sella turcica), which is prominent caudally as a well-developed ridge with two prominent lateral projections (dorsum sellae and clinoid processes). The dura mater (outer tough membrane of the meninges) is intimately associated with both the hypophysis and the sella turcica. The close association of gland, bone, and dura mater makes it difficult to remove the hypophysis without producing artifacts. In the dog, the adenohypophysis surrounds the neurohypophysis. Dorsally, the pars tuberalis surrounds the infundibulum and more ventrally, the pars distalis and pars intermedia surround the pars nervosa. The pars distalis is by far the largest part of the hypophysis. Cells of the pars distalis are traditionally classified as eosinophils, basophils, and chromophobes on the basis of their uptake of acidic or basic dyes. Immunocytochemical methods using specific hormonal antibodies now reliably distinguish the various cells responsible for secretion of each of the various hormones. The adenohypophysis produces a number of hormones: growth hormone (somatotropin), luteotropic hormone (prolactin), luteinizing hormone, follicle-stimulating hormone, thyrotropic hormone, adrenocorticotropic hormone, and melanocyte-stimulating hormone. Hormones produced elsewhere in the neurohypophysis but released into the bloodstream in the pars nervosa include antidiuretic hormone (vasopressin) and oxytocin (Capen, 1985).

Thyroid

The thyroid is a single gland consisting of two lobes that lie lateral to the first five to eight cartilaginous rings of the trachea (Miller et al., 1967). Occasionally, a glandular isthmus is found on the ventral surface of the trachea connecting the ventral poles of the two lobes. The thyroid is fairly loosely attached to the trachea. The size and shape of the lobes are not always identical. The thyroid arises from pharyngeal epithelium in close association with the aortic sac (a relationship that accounts for deposits of thyroid parenchyma anywhere from the larynx to the diaphragm).

The bud of thyroid epithelium expands and the attachment to the pharynx is narrowed to a slender stalk. A vestige of this stalk may persist on the midline as the thyroglossal duct. The thyroid expands laterally and fuses with the ultimobranchial bodies that arise from the fourth branchial pouch. The inclusion of parafollicular cells in the thyroid is the result of this fusion. Parafollicular cells (calcitonin-secreting cells, C cells) are of neural crest origin and migrate to the ultimobranchial body and from there to the thyroid. The thyroid is unique among endocrine glands because the final assemblage of hormone occurs outside the cell, within the lumen of the thyroid follicle (Capen, 1985). The manufacture and release of thyroid hormones, therefore, have an exocrine phase (synthesis and secretion of the protein, thyroglobulin, into the follicular lumen) and an endocrine phase (absorption of colloid from the lumen, release of hormones from the thyroglobulin molecule, and secretion of the hormones into the blood stream). The thyroid hormones are triiodothyronine (TO) and thyroxine (T4).

Parathyroid Glands

The parathyroid glands are paired and usually four in number. The parathyroids are entodermal in origin and derived from the third and fourth pharyngeal pouches (Miller et al., 1967).

Parathyroid III (commonly referred to as the external parathyroid) is a flattened oval body, 2–5 mm long, and most commonly found in the connective tissue about the cranial pole of a thyroid lobe. Its location may vary, but it is always external to the capsule of the thyroid.

Parathyroid IV (commonly referred to as the internal parathyroid) is generally smaller than the external parathyroid. The internal parathyroid is usually found beneath the capsule on the tracheal surface of a lobe, but it is also found deep within the thyroid.

Accessory parathyroids may be found within the thyroid, in the region of the larynx, the carotid sheath, anterior mediastinum, and within or associated with the thymus (Miller et al., 1967).

The parathyroid glands contain a single basic type of secretory cell, which secretes parathyroid hormone (parathormone, PTH).

Adrenal Glands

The adrenal glands are composed of two separate endocrine organs that differ in embryological origin, type of secretion, and function. The adrenal glands of the dog are generally flattened, bilobed, and located cranial and medial to the kidneys on either side of the aorta and posterior vena cava (Miller et al., 1967). The left adrenal lies further caudal than the right. The right adrenal is more irregular in shape, usually with a distinct hook or comma form. On section, the adrenal cortex is usually firm and yellow and the medulla is softer and is usually light red to brown.

The adrenal cortex develops from the cells of the coelomic epithelium. The chromaffin tissue and sympathetic ganglion cells of the adrenal medulla are derived from the neural crest. Neural crest cells invade the already formed cortical primordium, frequently leaving islands of medullary cells in the cortex or carrying groups of cortical cells into the medulla (Miller et al., 1967). Some of the medullary cells may not be incorporated in the adrenal gland and develop to form paraganglia and aortic and carotid bodies. The adrenal cortex is traditionally divided into three zones: glomerulosa, fasciculata, and reticularis. The zones may not be distinct. The outer zona glomerulosa (zona arcuata) represents about 15% of the cortex and is responsible for secretion of mineralocorticoid hormones. The middle zona fasciculata comprises about 70% of the cortex and is responsible for secretion of glucocorticoid hormones. The inner zona reticularis accounts for the remaining 15% of the cortex and is responsible for the secretion of sex steroids (Capen, 1985). The widths of the three zones vary and the demarcation between them may not be distinct. The adrenal medulla secretes epinephrine and norepinephrine.

Pancreas

The gross anatomy of the pancreas has been described with the digestive system. The pancreas is both an exocrine and endocrine gland. Cells responsible for the exocrine (digestive) secretions are the acinar cells. The cells of the pancreas responsible for hormone secretion (insulin and glucagon) are present within small spherical or oval islands (islets of Langerhans) dispersed throughout the organ. Islet cells are derived in common with acinar cells and are thus of entodermal origin.

Testis and Ovary

The anatomy of the testis and ovary is described with the genitourinary system. Testosterone is produced by interstitial cells (cells of Leydig) of the testis. Estradiol is produced by cells of the ovarian follicle. Progesterone is produced by cells of the corpus luteum.

Necropsy and Laboratory Techniques

A presentation of clinical signs of endocrine disease is beyond the scope of this chapter. The reader is referred to Ettinger (1989) as a source of clinical information. The endocrine glands should be handled carefully during dissection and weighing to minimize the production of artifacts. The thyroid and parathyroids should be removed while the larynx, trachea, and esophagus are in place and anatomical landmarks are undisturbed. Having all structures in place is particularly helpful when the thyroid is small or outside its normal location. Parathyroid glands should be included with the thyroid. The adrenal glands should be removed before the kidneys. The hypophysis may be removed from the sella turcica with minimal damage by first freeing the dorsum sellae by cutting through its base with small bone cutters. Second, grasp a clinoid process with fine forceps to support the hypophysis as the dura mater is cut with fine curved scissors to free the hypophysis from the basisphenoid bone. Third, remove the hypophysis from the cranial cavity attached to the dorsum sellae. Fourth, weigh the hypophysis by cutting the dura mater between the hypophysis and the bone to allow the hypophysis to drop upon a tared weighing boat.

Generally, a small strand of dura mater will be found on the hypophysis that may be grasped to transfer the gland to fixative or cassette. The hypophysis may be processed intact. If embedded with the dorsal (infundibular) surface down, three-step sections of the gland will usually result in a good representation of the pars distalis, pars intermedia, and pars nervosa.

During the trimming process, attempts should be made to include at least one parathyroid gland with each lobe of the thyroid. Both lobes of the thyroid should be represented in stained sections. Adrenal glands should be cut parasagittally. The thicker segment from each gland is embedded. The block may be rough cut on the microtome to obtain nearly full width representations of the cortex and medulla in stained sections.

Pathology

Of all the endocrine glands, potentially significant lesions are most likely to be seen within the thyroid gland. Cysts are frequently found (less frequently reported) in the hypophysis, parathyroid, and thyroid.

Hypophysis (Pituitary Gland)

Cysts are often observed in the hypophysis of dogs. Cystic remnants of the craniopharyngeal duct are frequently found at the periphery of the pars tuberalis and pars distalis. Most of the cysts

are microscopic, but a few are visible grossly. The cysts are lined by cuboidal to columnar epithelium, often ciliated, and contain mucin. Maita et al. (1977) and Oghiso et al. (1982) reported cysts in the anterior lobe of the hypophysis. Pituitary cysts were reported in 24%–26% of young beagles by Glaister (1986), in 44 (7%) of 647 dogs on 39 studies by Hottendorf and Hirth (1974), and in 3 (8%) of 37 untreated beagles by Pick and Eubanks (1965). Inflammation as reported was limited to granulomas with 1 (0.1%) of 647 dogs affected (Hottendorf and Hirth, 1974). Barron and Saunders (1966) reported the occurrence of Toxocara granulomas in the pituitary.

Parathyroid Glands

Small cysts (apparently from remnants of the duct connecting thymic and parathyroid primordia) may be found occasionally within or near the parathyroid. Parathyroid cysts are usually multiloculated, lined by cuboidal to columnar epithelium, often ciliated, and contain densely eosinophilic material. Parathyroid cysts occurred in 1% of young beagles (Glaister, 1986). Hyperplasia of parathyroid cells was observed in 7 (1%) of 647 dogs (Hottendorf and Hirth, 1974).

Thyroid

Two potentially important changes in the thyroid of laboratory dogs are idiopathic follicular atrophy and lymphocytic thyroiditis. Minor changes frequently seen within the thyroid include disassociation of follicular cells (probably artifactual), minute intrafollicular corpora amylaceae–like bodies, and the presence of brown pigment (lipofuscin) in follicular cells.

Idiopathic follicular atrophy is a progressive loss of follicular epithelium and replacement by adipose cells. One lobe may be affected more than the other. Severely affected lobes are difficult to locate at necropsy. Parafollicular cells are not affected and remain in the adipose tissue. Dogs with idiopathic thyroid atrophy may be hypothyroid. Hypothyroidism may also be seen in dogs with lymphocytic thyroiditis. Atrophy of the thyroid was observed in 10 (2%) of 647 dogs (Hottendorf and Hirth, 1974). Follicular atrophy was reported by Oghiso et al. (1982).

Lymphocytic thyroiditis in dogs appears to have an immunological basis and familial occurrence in beagles. Glands with lymphocytic thyroiditis may be enlarged, but they may also be normal or even reduced in size. Histological alterations consist of multifocal to diffuse infiltrates of lymphocytes, plasma cells, and macrophages. Lymphoid nodules may be present. Thyroid follicles are usually small and may be disrupted and contain degenerate follicle cells, lymphocytes, and plasma cells. Lymphocytic thyroiditis in the beagle has been extensively investigated (Tucker, 1962; Mawdesley-Thomas and Jolly, 1967; Mawdesley-Thomas, 1968; Beierwaltes and Nishiyama, 1968; Musser and Graham, 1968; Fritz et al., 1970; Mizejewski et al., 1971). Lymphocytic thyroiditis was reported by Oghiso et al. (1982). Chronic lymphocytic thyroiditis was observed in 22 (3%) of 647 dogs on 39 studies (Hottendorf and Hirth, 1974).

Unspecified thyroiditis occurred in 3 (8%) of 37 untreated beagles (Pick and Eubanks, 1965). Barron and Saunders (1966) reported the presence of *Toxocara* granulomas in the thyroid.

Ultimobranchial duct cysts are frequently seen within the thyroid. They are derived from remnants of the ultimobranchial body and have a keratinized squamous epithelial lining (Capen, 1985). Ultimobranchial duct cysts was observed in 12 (2%) of 647 dogs on 39 studies (Hottendorf and Hirth, 1974). Cysts occurred in the thyroids of 1%–2% of young beagles (Glaister, 1986). Young beagles have varying numbers of parafollicular or C cells in the thyroid. Occasionally, the number or focal concentration appears adequate to justify a diagnosis of C-cell hyperplasia. C-cell hyperplasia occurred in 8%–9% of young beagles (Glaister, 1986). Interfollicular cellularity was reported by Oghiso et al. (1982). Follicular epithelial hyperplasia was observed by Maita et al. (1977). Lymphoid hyperplasia in the thyroid was observed in 2% of young beagles (Glaister, 1986).

Focal squamous metaplasia was reported in 5 (0.8%) of 647 dogs (Hottendorf and Hirth, 1974). Ectopic thymus was observed in 1%–2% of young beagles (Glaister, 1986). Additional minor changes frequently seen within the thyroid include disassociation of follicular cells (probably artifactual), minute intrafollicular corpora amylaceae–like bodies, and the presence of brown pigment (lipofuscin) in follicular cells.

Adrenal Glands

As noted earlier, the width of the various layers of the adrenal cortex may vary. The zona glomerulosa may be thin or seem to be absent for substantial distances. Cortical cells may be found in the medulla and medullary cells may be found in and on the cortex (all explainable in the embryological development of the gland). Similarly, accessory adrenal cortical tissue is also common outside the adrenal capsule (Sato et al., 2012). Focal cortical hyperplasia was observed in 19 (3%) of 647 dogs on 39 studies (Hottendorf and Hirth, 1974). Nodular hyperplasia of the adrenal cortex was reported by Oghiso et al. (1982). Appreciable differences in cortical cell vacuolation can be observed from animal to animal. Vacuolation related to accumulation of large lipid droplets is seen frequently in zona glomerulosa (Sato et al., 2012). This normal vacuolation must be distinguished from fatty degeneration. Fatty degeneration was reported by Oghiso et al. (1982). Minor focal inflammation in the adrenal occurred in 5 (0.8%) of 647 dogs (Hottendorf and Hirth, 1974). Occasionally, extramedullary hematopoiesis of erythrocytic or granulocytic series can be seen in the adrenal sinusoids (Sato et al., 2012). Yellow-brown pigment deposition, considered to be lipofuscin, can be seen in the cytoplasm of the cortical cells predominantly at the corticomedullary junction (Sato et al., 2012).

Urogenital System

Anatomy and Histology

The urogenital system is made up of the urinary organs (kidneys, ureters, urinary bladder, and urethra), the male genital organs (scrotum, testes, epididymides, deferent ducts, prostate, and penis) and the female genital organs (ovaries, oviducts, uterus, vagina, and vulva).

Kidneys

The kidney performs metabolic, humoral, and excretory functions. The excretory function produces a fluid (urine) that is conducted from the kidney by fibromuscular tubes (ureters) to a storage reservoir (urinary bladder), where the fluid accumulates and is periodically discharged through a single tube (urethra) to the exterior.

The kidneys are bean-shaped glands located in the sublumbar area, one on each side of the aorta and posterior vena cava. The right kidney is usually located slightly anterior to the left and is more firmly secured in its position. A whole kidney cut in a midsagittal plane has two distinct zones (cortex and medulla). The cortex is the darker outer zone lying beneath the renal capsule. The medulla is the remaining lighter zone shaped like an inverted pyramid.

The apex of the pyramid is the papilla, which projects into the expanded end of the ureter (renal pelvis). The renal pelvis lies within an opening in the medial border of the kidney called the renal hilus (renal sinus). In addition to the renal pelvis, the hilus contains adipose tissue, branches of the renal artery, vein and lymphatics, and nerves. Since the dog has only one papilla, it is classified as a unilobular or unipyramidal kidney; however, the single pyramid is actually the result of several pyramids fusing during development (Brown, 1976).

The nephron is the functional unit of the kidney and has six morphologically distinct segments: renal corpuscle, convoluted and straight portions of the proximal tubule, thin segment, and straight

and convoluted portions of the distal tubule. The distal tubules join collecting tubules. Collecting tubules join with other collecting tubules and finally converge into large collecting ducts (papillary ducts) that open onto the apex of the papilla. The renal corpuscles and convoluted portions of the proximal and distal tubules are located in the cortex. The thin segment and straight portions of the proximal and distal tubules are located in the medulla. The latter three segments form a loop called the loop of Henle. In unipyramidal kidneys, segments of Henle's loops are so regularly arranged that a separation of medulla into outer and inner zones is visible to the naked eye. The boundary between the zones is the junction of the ascending limb of the thin segment and the straight portion of the distal tubule. The junction of the straight portion of the proximal tubule with the descending thin limb subdivides the outer zone into inner and outer bands, which are also visible to the naked eye (Brown, 1976).

Ureters

The ureters are slightly flattened tubes that begin at the renal pelvis and enter the urinary bladder by separate orifices near the neck of the bladder.

Urinary Bladder

The urinary bladder is a hollow musculomembranous organ whose form, size, and position vary according to the volume of urine it contains. The near full capacity of the bladder in the beagle is about 150 mL (Andersen, 1970). The urinary bladder is divided into a neck region (connecting with the urethra), a blunt cranial end (fundus), and a body portion between the neck and fundus (Miller et al., 1967). Internally, a triangular area near the neck is termed the trigone. The base of the trigone is a line connecting the ureteral openings; the apex is the urethral orifice.

Urethra

The male urethra is the canal that carries urine and seminal secretions to the exterior. It is divided into prostatic, membranous, and penile portions. The female urethra extends from the urinary bladder to enter the vulva just caudal to the vaginovulvar junction.

Male Genital Organs

Scrotum

The scrotum is divided by a median septum into two cavities, each of which is occupied by a testis, its associated epididymis, and a distal part of the spermatic cord. The scrotum functions as a temperature regulator for the testes, made possible by its thin gland-rich skin, lack of subcutaneous fat, and an ability to contract toward the body (by means of the cremaster muscles) or relax away from the body.

Testis

The canine testis is oval and is thicker dorsoventrally than from side to side. The size (weight) of testes of young adult beagles varies so greatly as to be significant concern in short-term toxicology studies. James and Heywood (1979) demonstrated the magnitude of the weight variation in beagles on 13-, 26-, and 52-week (and longer) studies (Table 8.12). In their experience, male beagles attain full sexual maturity between 35 and 40 weeks of age (based on testicular size, semen evaluation, hormonal profiles, and quantitative histometric analysis of spermatogenesis).

Table 8.12   Testicular Weight in Beagles under 2 Years of Age

Number of Dogs	Mean Age (Weeks)	Mean Body Weight (kg)	Mean Testicular Weight (g)		% of Dogs with Testicular Weight in Range (g)		
					0.0–19.9	20.0–29.9	T ≥ 30
42	37 ± 4	11.9 ± 1.8	20.7 ± 6.1	4.8	35.7	52.4	7.1
38	46 ± 2	15.6 ± 2.0	23.6 ± 5.0	0.0	26.3	63.2	10.5
41	73 ± 4	12.4 ± 2.0	25.0 ± 6.6	2.4	17.1	56.1	24.4

The cut surface of a normal testis bulges. The color of the testis in young beagles is light pink to tan (depth of color increases with age due to increased pigmentation of the interstitial cells). The testis is subdivided into numerous lobules by delicate connective tissue septa that extend from a core of connective tissue (mediastinum testis) to the capsule of the testis (tunica albuginea). Ducts, blood vessels, lymphatics, and nerves enter and leave the testis through the mediastinum. Each of the lobules contain one or more convoluted sperm-producing tubules (seminiferous tubules) that empty at both ends into straight tubules (tubuli recti) that connect with a series of epithelial lined channels (rete testis) in the mediastinum. Spermatozoa are swept out of the seminiferous tubules, through the rete testis, and into the epididymis in a fluid secreted by the sustentacular cells (Sertoli cells). The interstitial cells (Leydig cells) are the endocrine (testosterone-producing) cells of the testis.

Epididymis

The epididymis consists of the ductuli efferentes and the much longer and highly tortuous ductus epididymidis. The ductuli efferentes emerge from the mediastinum to connect the rete testis with the ductus epididymidis. The epididymis lies along the dorsolateral surface of the testis. For descriptive purposes, the epididymis is divided into three portions. The initial portion located near the cranial extremity of the testis and into which the ductuli efferentes empty is called the head of the epididymis (caput epididymis). The portion of the head with the ductuli efferentes is called the initial segment. The main length of the epididymis is the body (corpus epididymis), and the segment attached to the caudal extremity of the testis is the tail (cauda epididymis). The epididymis is the maturation and storage site for the spermatozoa. The ductus epididymidis is subdivided further, with each subdivision apparently fulfilling a specific function in the maturation process of the sperm.

Ductus Deferens

The ductus deferens (deferent duct) is a thick-walled tube that is continuous with the tail of the epididymis and extends to the prostatic urethra. Its initial portion is located within the spermatic cord and surrounded by the veins of the pampiniform plexus, arteries, lymph vessels, nerves, and smooth muscle.

Prostate Gland

The prostate gland (the only accessory male sex gland in the dog) is an ovoid musculoglandular organ that completely surrounds the proximal portion of the ureter. The prostate is composed of two portions: external and internal. The external portion forms most of the dog's prostate and consists of two large bilateral lobes. The internal portion consists of a few small glands scattered along the urethra. Trabeculae divide the two external lobes into lobules in which the glandular elements are most prevalent near the periphery. Secretion from prostate glands enters the urethra by way of numerous excretory ducts. The paired ductus deferens enter the dorsal surface of the prostate and run caudoventrally on either side of the median plane to open into the dorsal surface of the prostatic urethra.

Table 8.13 Prostate Weight in Beagles under 2 Years of Age

Number of Dogs	Mean Age (Weeks)	Mean Body Weight (kg)	Mean Prostate Weight (g)	% of Dogs with Prostate Weight in Range (g)			
				<5.0	5.1–10.0	10.1–20.0	>20
42	37 ± 4	11.9 ± 1.8	3.3 ± 1.6	84.4	15.6	0.0	0.0
38	46 ± 2	15.6 ± 2.0	6.5 ± 2.3	23.4	66.1	10.5	0.0
41	73 ± 4	12.4 ± 2.0	8.1 ± 2.9	9.8	63.4	26.8	0.0

James and Heywood (1979) found incomplete prostate development in beagles 9–10 months of age and demonstrated a wide range of prostate weights in control beagles on short-term toxicity studies (Table 8.13).

Penis

The male copulatory organ (penis) is composed of three principal parts: root, body, and distal free part (glans penis). The glans penis is mostly enveloped in stratified squamous epithelium and, when not erect, is entirely withdrawn into a tubular sheath of integument (prepuce). The mucosa of the penile urethra is lined by transitional epithelium except near the external urethral opening, where it changes to stratified squamous epithelium similar to that covering the penis.

Female Genital Organs

The female genital organs consist of the ovaries, oviducts, uterus, vagina, and vulva. The ovaries, oviducts, and uterus are attached to the walls of the abdominal and pelvic cavities by folds of peritoneum called broad ligaments. Each broad ligament contains an ovary, oviduct, and uterine horn (plus vessels, nerves, and fat).

Ovary

The ovary is an ovoid gland situated within a fossa of the peritoneum (ovarian bursa) and supported by the mesovarium (cranial portion of the broad ligament) and the suspensory ligament of the ovary. The ovary is completely enclosed by the ovarian bursa except for a narrow slit, 2–15 mm long, located on the medial side. The structure of the ovaries varies with age and the phase of the sexual cycle.

The ovary is composed of a cortex and medulla that contains a prominent rete ovarii. The cortex consists of a connective tissue stroma, which contains blood vessels, lymphatics, follicles, and corpora lutea. The surface of the cortex is smooth in immature ovaries.

Oviducts

The oviducts (uterine tubes) are tortuous structures that extend from the region of the ovary to the uterine horns. In the bitch, the oviduct almost completely encircles the ovary. The large funnel-shaped ovarian end of the duct is called the infundibulum and is located near the slit in the ovarian bursa.

Uterus

The uterus is a Y-shaped tubular organ that communicates with the oviducts cranially and the vagina caudally. The uterus consists of a neck (cervix), body (corpus), and two horns (cornua). In the

bitch, the uterine body is short and the horns relatively long and straight. The right horn is usually longer than the left. The size and shape of the uterus varies according to age and the stage of the sexual cycle. The uterine horns unite at the body. The body extends from the point of convergence of the uterine horns to the cervix. The wall of the uterus consists of three layers: mucosa (endometrium), muscularis (myometrium), and serosa (perimetrium). The endometrium is the thickest of the three layers and consists of three zones: crypt, intermediate, and basal (McEntee, 1990). The crypt zone has numerous short, epithelial-lined recesses. The intermediate zone contains uterine glands, but it is predominantly connective tissue. The uterine glands branch, coil, and terminate in the basal zone.

Vagina

The vagina is the highly dilatable musculocutaneous canal extending from the uterus to the vulva. Cranially, the vagina is limited by the cervix, which may protrude up to 1 cm into the vagina. Caudally, the vagina ends just cranial to the urethral opening. Flat longitudinal folds extend throughout the length of the vagina. The tunica mucosa is nonglandular stratified squamous epithelium.

Vulva

The vulva is the external genitalia of the bitch and consists of the vestibule, clitoris, and labia. The vestibule is the space connecting the vagina with the external genital opening and is the largest part of the vulva. The vestibule is the common opening for the genital and urinary tracts. The clitoris (homologue of the male penis) is located in the extreme caudal region of the vestibule near the ventral commissure of the vulva. The labia (lips) form the external boundary of the vulva. The vestibule is lined by stratified squamous epithelium and contains small, mucus-producing vestibular glands. Numerous lymph follicles are present in the vestibular mucosa and may be large enough to be seen grossly. The labia are covered with stratified squamous epithelium and are rich in sebaceous and tubular sweat glands.

Estrous Cycle

Puberty is the age at which first estrus occurs in the bitch and varies among breeds and within breeds. The range is usually given as 6–12 months. The average age for first estrus in the laboratory beagle appears to be about 12 months, with a range of 10–14 months (Andersen, 1970; Sekhri and Faulkin, 1970; Sokolowski, 1973). Most female beagles will be sexually immature when started on toxicology studies. At termination of short-term studies, they may still be sexually immature or be in any stage of their first estrous cycle. The sexual stage of the bitch at termination of a study impacts the gross and microscopic appearance of the sex organs (and mammary gland) and the weights of the ovaries, uterus, and conceivably the hypophysis and adrenal glands. Traditionally, the cyclical changes occurring in the reproductive system of the bitch have been divided into four phases: proestrus, estrus, metestrus, and anestrus. This classification has deemphasized the important extended luteal phase of the cycle and obscured several events, which occur during the estrus phase of the cycle. Recently, several workers (Cupps et al., 1969; McDonald, 1969; Holst and Phemister, 1974) have reexamined the traditional classification and suggested modification. Phemister (1974) summarizes the revised view as follows:

> Proestrus remains the phase of rapid follicular growth and rising estrogen levels when there is swelling of the vulva, generalized congestion of the genital tract, and a sanguineous vaginal discharge. As a rule, proestrus lasts about 9 days. Estrus is the period of sexual receptivity, usually lasting 7 to 10 days. In most bitches the initial day of estrus coincides approximately with a surge of luteinizing hormone (LH) from the pituitary gland. The LH surge is followed by ovulation 2 days later, usually on about the 3rd day of estrus. Metestrus, as the term is used for other species, defines the brief period

when the corpus luteum is being formed and becoming functional. In the bitch this phase lasts for about 4 days and occurs entirely within the period of acceptance (estrus). By 4 days after ovulation, the genital system is dominated by luteal progesterone. By definition this phase of progesterone dominance is diestrus. Its onset is signaled by an abrupt change in vaginal cytologic characteristics: from predominantly large cornified, superficial squamous cells to noncornified, small intermediate and parabasal cells. On an average, diestrus begins 2 to 3 days before the end of estrus, and based on hormonal data, lasts for 2 to 3 months, or even longer if morphologic data are used. Following diestrus, the bitch enters a period of reproductive quiescence, anestrus, which lasts for 3 months or more.

Metestrus in the revised classification describes the period more in accordance with its use in other species and emphasizes the fact that corpora lutea form and function for comparable periods (2–3 months) in a bitch whether she is pregnant or not pregnant. In other words, every nonpregnant bitch experiences a period of pseudopregnancy (pseudocyesis) to some degree. When the bitch's appearance and behavior closely mimic pregnancy, the pseudopregnancy becomes a clinical problem.

Necropsy and Laboratory Techniques

Because of the large functional reserve of the kidney, renal diseases may or may not be associated with renal dysfunction. Two-thirds to three-fourths of the renal parenchyma must be functionally impaired before clinical signs develop in chronic renal failure (Osborne et al., 1983). Urinary enzymes have been useful in dogs for detecting acute renal damage, but less helpful for chronic damage. Increases in brush border enzymes, including gamma-glutamyl transferase and alkaline phosphatase, have been associated with proximal tubular damage, whereas increases in N-acetyl-beta-D-glucosaminidase have been observed in the early stages of renal papillary necrosis (Clemo, 1998). Functional abnormalities of the kidney can be clinically manifested in a number of ways, including polyuria (formation and elimination of large quantities of urine), oliguria (decrease in the rate of formation and/or elimination of urine), anuria (lack of urine formation/lack of urine elimination), polydipsia (excessive thirst), weight loss, anorexia, vomiting, diarrhea, dehydration, edema, stomatitis, weakness, and depression. Cloudy urine or hematuria may indicate disease of the urinary bladder. It is unlikely that signs of a reproductive system disorder would be detected in short-term toxicology studies. At postmortem, the initial examination of the urogenital system should be done with all organs of the system in place.

Kidney

The kidneys should be approximately equal in size. The normal cortex is brownish red and the medulla white to pink. The kidney should be cut into multiple transverse sections and the surfaces of each examined. One or two sections should be taken from the midpyramidal region and placed in fixative. Proximal tubular epithelium autolyzes rapidly and kidneys should be placed into fixative as early as possible. Both the immature and mature kidney of the beagle have been studied in detail and much is known about normal morphometry of the organ (Stuart et al., 1975; Eisenbrandt and Phemister, 1977, 1978 1979, 1980; Jaenke et al., 1980).

Ureters and Urinary Bladder

Usually, the ureters need only to be examined externally in situ. The urinary bladder should be removed, incised longitudinally and everted, and the mucosal surface examined. The entire bladder should be fixed. Usually, the bladder contains some urine at necropsy. Uncontaminated urine specimens may be gotten by puncturing the undisturbed bladder with a hypodermic needle (avoiding blood vessels) and withdrawing urine into a syringe.

Prostate, Scrotum, and Epididymis

The prostate should be removed and its internal surface examined by one or more transverse cuts (some prostates are so small that bisection is adequate). At least two sections should be fixed. The scrotum should be incised, the scrotal ligaments severed, the testes removed, and the vaginal tunics surrounding the spermatic cords incised to the level of the abdominal wall. After examining the structures of the spermatic cords and the surfaces of the testis and epididymis, the testis and epididymis are freed. The epididymis should be dissected from the testis before the testis is weighed. The epididymides may be fixed intact. The testis should be sliced before fixation to verify that the cut surface bulges. (Failure to bulge indicates degeneration of seminiferous tubules.) The incisions should be made with a very sharp knife. McEntree also recommends samples be taken from the head extremity, middle part, and caudal extremity. Bouin's is the recommended fixative for the testis.

Ovaries and Uterus

The ovaries and uterus are usually weighed in toxicology studies. As with the testis and prostate, the weight of the female organs varies according to sexual maturity and, additionally, upon the phase of the estrous cycle. Reproductive organs should be examined in detail in studies of compounds with known or potential effects upon the reproductive system. Less attention may be paid to the reproductive tract in other studies; nevertheless, it seems appropriate that some observations be made to establish the probable sexual state of a bitch at necropsy and to have these observations to correlate with microscopic findings and organ weight data.

Gross and Microscopic Appearance of Female Genital Organs at Necropsy

The gross and microscopic appearance of the female genital organs of the beagle during the various phases of the estrous cycle have been described (Andersen, 1970; Sokolowski et al., 1973; Sokolowski, 1977; Sato et al., 2012).

Vulva

The external genitalia swell and discharge a sanguineous fluid at the onset of proestrus. (The sanguineous discharge of proestrus and estrus is not due to mucosal hemorrhage but due to extravasation of erythrocytes by diapedesis through the endometrium and into the lumen of the uterus and vagina.) The thickening of the labia and wall of the vestibule is due to congestion and edema that account for the warm feeling of the external genitalia from which the expression "in heat" is derived. The vestibular mucosa remains unwrinkled during proestrus and estrus, but lymph follicles may become prominent.

Vagina

The vaginal mucosa shows extensive wrinkling beginning at proestrus and continuing into estrus. (The normal longitudinal folds are exaggerated and in turn irregularly subdivided by numerous transverse wrinkles.) The wrinkling decreases later in estrus and by early metestrus (diestrus), the folds have lost their wrinkled appearance. The cervix protrudes prominently into the vagina during estrus, and its surface is marked by numerous folds. The cervical canal is patent throughout estrus and early diestrus. By the end of diestrus and throughout anestrius, the cervical canal is almost completely sealed.

Uterus

Enlarged blood vessels appear in the broad ligaments and in the perimetrium with proestrus and are present throughout estrus. The uterine wall thickens owing to congestion, edema, and proliferation of uterine glands. The uterus attains maximum nonpregnant size 20–30 days post ovulation and exhibits a characteristic "corkscrew" appearance. At 60 days postovulation, the uterus is about the same size as in proestrus. The uterus never returns to the size seen in immature bitches.

Oviduct

During estrus, a small mass of red tissue protrudes from the slit-like opening of the bursal sac. The tissue is hyperemic and edematous fimbriae, which almost completely close the slit.

Ovary

The ovaries of immature bitches are small, smooth, and have no follicles larger than 1 mm in diameter. A definite cortex and medulla are present at 6 months. On the first day of proestrus, follicles may be up to 4 mm in diameter and up to 14 mm in diameter just prior to ovulation. Ovarian weight is greatest at approximately the time of ovulation (presumably due to the size of follicles and the luteinizing that is occurring). Luteinizing begins immediately after follicles rupture, and it is not unusual to find follicles of varying size and corpora lutea in the same ovary at estrus. Corpora lutea are most numerous and fully developed about 10 days after ovulation. Corpora lutea begin to degenerate at about 20 days postovulation and by 60 days postovulation have undergone fatty degeneration and appear nonfunctional. Corpora lutea are bright salmon pink from the time of ovulation until 10 days after ovulation. They then gradually yellow and are light tan about 60 days after ovulation. No remnants of corporea lutea (corpora albicantia) are seen in ovaries during the first estrous cycle.

Mammary Gland

Gross changes are imperceptible in the mammary gland until shortly after ovulation, when a bluish plaque can be observed at the base of each teat. Thereafter, the mammary glands enlarge and regress during the metestrus (diestrus) stage of the estrous cycle.

Pathology

Many changes have been reported for the kidney and male genital organs. In contrast, few observations have been made for the female genitalia.

Nonneoplastic Findings, Spontaneous

Kidney

Inflammation of the kidney is a frequent finding in dogs. The inflammation may be either primary or secondary and affects various tissue components resulting in many disease entities being reported. Inflammation of the kidney without identifying specific affected components can also be termed nephritis. Minor focal nonspecific inflammation occurred in 76 (12%) of 647 dogs (Hottendorf and Hirth, 1974). Leukocytic foci were observed in 14% of young beagles (Glaister, 1986). Focal embolic nephritis was reported in 1 of 647 dogs (Hottendorf and Hirth, 1974).

Inflammation of the glomerulus can also be termed glomerulitis, glomerulonephritis, and glomerulosclerosis. Glomerulitis and glomerulonephritis were reported in 2 (0.3%) of 647 dogs (Hottendorf and Hirth, 1974). Local or diffuse mesangial proliferation and thickened and wrinkled glomerular basement membranes are not an unusual finding in clinically healthy, nonproteinuric laboratory beagles (Stuart et al., 1975). Periglomerular sclerosis may also be present. With time, the intercapillary sclerosis generally increases in severity and may be associated with intermittent or persistent proteinuria. A progressive alteration of the renal glomerulus called progressive intercapillary glomerulosclerosis was described by Guttman (1970). This change is seen as early as 6 months of age and consists of thickening of basement membranes and increase in the mesangial matrix. Immature glomeruli can be found in the outer layer of the cortex and reflect delayed maturation of the glomeruli in this region. These glomeruli have a small profile with prominent epithelial cells and indistinct capillary formation. Small glomeruli are also present in rare cases of renal dysplasia along with immature renal tubules and dilatation of collecting tubules. The glomerulus and interstitium are also rarely affected by osseous metaplasia (Sato et al., 2012).

Inflammation of the interstitium usually termed interstitial nephritis or chronic interstitial nephritis is common. Interstitial nephritis was reported by Oghiso et al. (1982). Focal interstitial nephritis was recorded in 6% of young beagles (Glaister, 1986). Chronic interstitial nephritis occurred in 6 (0.9%) of 647 dogs (Hottendorf and Hirth, 1974). Inflammatory cell infiltration occasionally occurs surrounding focal regenerative changes of damaged tubules, which appear as basophilic tubules with a high nuclear density in the cortex or outer stripe of the renal medulla (Sato et al., 2012).

Inflammation of the pelvis, also termed pyelitis and pyelonephritis, is another common finding.

Pyelitis usually with minor degrees of mononuclear infiltration of the lamina propria and epithelium of the renal pelvis was observed in 4%–7% of young beagles Glaister (1986). Pyelonephritis is a nephritis that arises in the pelvis and spreads upward to adjacent areas of the kidney. Pyelonephritis was reported by Oghiso et al. (1982). Pyelitis or pyelonephritis occurred in 12 (2%) of 647 dogs (Hottendorf and Hirth, 1974). Pyelitis and pyelonephritis were also reported by Pick and Eubanks (1965) and can feature lymphoid follicle formation as a component of the inflammatory response. Focal hyperplasia of the pelvic epithelium can occur in association with pyelitis (Sato et al., 2012).

Granulomatous inflammation or granulomas are frequent findings. Barron and Saunders (1966) reported the kidney as a common site for *Toxocara* granuloma. Cortical *Toxocara* granulomas were reported in 4% of young beagles Glaister (1986). Cortical granulomas were observed in 2 (5%) of 37 dogs (Pick and Eubanks, 1965). Granulomas occurred in 11 (2%) of 647 dogs (Hottendorf and Hirth, 1974). Toxocar granuloma were also recorded in the kidney by Maita et al. (1977).

Mineralization of the kidney is very common in dogs and is usually seen in the form of clumps of basophilic granules adjacent to and in the lumen and lining of collecting tubules. The incidence is probably higher than the reported 50%. Collecting tubule calcification was reported by Pick and Eubanks (1965) and calcification by Oghiso et al. (1982). Microcalculi of the renal medulla were found in almost 50% of the 647 males and females (Hottendorf and Hirth, 1974). Mineralization of renal papilla was observed in both males and females (Glaister, 1986). Degenerative changes of the glomeruli and tubules were mentioned but these changes were not described by Oghiso et al. (1982). Oghiso et al. (1982) also reported albuminous urinary casts. Focal hyaline casts are sometimes observed in the distal segment or collecting tubule in the outer medulla (Sato et al., 2012). Occasionally, one or more lobules of the glomerular tuft will be filled with large foam cells, which with appropriate stains are shown to contain fat. The condition is known as glomerular lipidosis and has no known functional significance. Mesangial cells may contain lipid droplets accompanied by hyaline droplets. Lipid droplets are also known to accumulate in the epithelia of the straight portion of the proximal tubules in female dogs (Sato et al., 2012).

Hottendorf and Hirth (1974) reported the occurrence of hydronephrosis in their 647 dogs. Acidophilic rectangular or cubic crystalline intranuclear inclusions (identical in appearance to those seen in hepatocytes) are also commonly found in nuclei of cells lining proximal and

distal tubules (Oghiso et al., 1982). Basophilic or eosinophilic inclusion bodies have also been reported in the cytoplasm of collecting tubule epithelium and are often accompanied by pyelitis. Deposits of brown pigment considered to be lipofuscin can be seen in proximal tubular epithelium (Sato et al., 2012).

Unilateral renal agenesis is occasionally found in laboratory beagles (Robbins, 1965; Vymetal, 1965; Hottendorf and Hirth, 1974) and was observed in adult breeding colony beagles (Fritz et al., 1976). Four (0.4%) of 1000 dogs were affected (Hottendorf and Hirth, 1974). This condition is not detected during routine physical examinations. The ureter may or may not be missing. The developed kidney is usually about twice normal size (weight) and histologically of normal appearance, suggesting that the increased size is due to an absolute increase in the number of nephrons. Postnatal nephrogenesis is a characteristic of the canine kidney. Eisenbrandt and Phemister (1979) have shown that nephrogenesis continues through the first 8–10 days of life in the beagle, a fact that probably accounts for the normal histological appearance of the single enlarged kidney seen in unilateral renal agenesis.

Urinary Bladder

Calculi and cystitis were seen infrequently in the urinary bladder, more often in males (Hottendorf and Hirth, 1974). Cystitis occurred in 27 (23 males and 4 females) of 647 dogs (Hottendorf and Hirth, 1974). Catheterization may be the cause of some instances of prostatitis and cystitis. Detrusor myopathy is a condition characterized by degenerative lesions in the urinary bladder muscle tunics that occurred in 59 of 449 (13%) young beagles. Both sexes were affected equally. Arteritis and periarteritis were reported in 10 of the 59 dogs with detrusor myopathy (Cain et al., 2000). Calcification of the round ligament of the bladder (remnant of the umbilical artery of the fetus) was seen, but not listed as a significant finding (Hottendorf and Hirth, 1974). Calculi were reported as an uncommon finding in the urinary bladder. Five animals were grossly affected in 1000 dogs (Hottendorf and Hirth, 1974).

Urethra

Calculi are an unusual finding in the urethra with one animal grossly affected in 1000 dogs (Hottendorf and Hirth, 1974).

Testis

Focal atrophy of seminiferous tubules was reported in 11 (3%) of 326 dogs by Hottendorf and Hirth (1974) and in 5% of young beagles by Glaister (1986). Testicular degeneration occurs naturally in the dog and may be focal or diffuse and unilateral or bilateral. Early degenerative lesions consist of loss of primordial germ cells that may appear within the lumen of the seminiferous tubule as individual cells or as multinucleated giant cells. As the degeneration advances, more germinal cells are lost and tubules may be lined only by sustentacular cells (Sertoli cells). Segmental lesions of Sertoli-cell-only tubules are common and may represent segmental hypoplasia of the seminiferous tubules during development (Sato et al., 2012). Sato et al. (2012) also note that sporadic vacuolation of Sertoli cells is a common finding. Intratubular giant cells were observed in the seminiferous tubules of 9 (3%) of 326 untreated young beagles (Hottendorf and Hirth, 1974). Lymphocytic orchitis has been observed in beagle colonies and occur in laboratory beagles, usually associated with lymphocytic thyroiditis. The lymphocytic infiltration may be diffuse, aggregated, or nodular (with germinal centers) and is commonly associated with focal or diffuse degeneration and atrophy of seminiferous tubules. The epididymis may be involved (Fritz et al., 1976). Orchitis can be caused by *B. canis* infections (Jubb et al., 1992). Seminiferous tubular damage can result in the formation of

spermatocoeles or retention of spermatids in the seminiferous tubules (Sato et al., 2012). Interstitial cell hyperplasia is rarely seen in untreated young beagles. It was noted in 1 (0.3%) of 326 dogs (Hottendorf and Hirth, 1974).

Epididymis

Inflammation of the epididymis was reported in 1 of 326 male dogs by Hottendorf and Hirth (1974). Epididymitis can be caused by *B. canis* infections (Jubb et al., 1992). Lymphocytic epididymitis often associated with lymphocytic orchitis and thyroiditis is characterized by lymphocytic infiltrations (Fritz et al., 1976). Spermatic granulomas are inflammatory lesions that occur in the efferent ductules and epididymis. Intratubular granulomas were observed in the caput epididymis of three clinically normal dogs that were associated blind-ending efferent ducts. While spermatic granulomas can be induced by trauma, infection, or toxins, spontaneous granulomas due to blind-ending ductules should be considered in the differential (Foley et al., 1995). James and Heywood (1979) report spermatocoele granuloma (spermatic granuloma) and inflammation for the epididymis. Intraluminal cellular debris can be prominent in cases of seminiferous tubular damage. Cystic spaces are sometimes observed within the tubular epithelium, which are actually intraepithelial lumens lined by ciliated cells. Epididymal epithelia can also be obscured by mineral deposition (Sato et al., 2012).

It is normal to find intranuclear, eosinophilic, PAS-positive inclusions in the epididymal epithelium of the dog (McEntree, 1990). The significance of the inclusions is unknown. Normal epididymal cells also contain granular, yellow to yellow-brown pigment. The epididymides of immature beagles contain ducts with narrow lumens lined by small, flat epithelia, and immature fibrous tissue with high nuclear density comprises the interstitium (Sato et al., 2012).

Prostate

Subclinical prostatitis is common in the dog. The inflammation is usually minimal in young adult beagles. Prostatitis was reported in 124 (38%) of 326 males from 39 studies (Hottendorf and Hirth, 1974). Chronic inflammation of the prostate was reported also by Maita et al. (1977) and James and Heywood (1979). Collection of urine by catheterization may be the cause of some prostatitis. Prostatitis can be caused by *B. canis* infections (Jubb et al., 1992). Minor accumulations of lymphocytes (leukocytic foci) to large lymphoid aggregates with germinal center formation accompanied by interstitial fibrosis and epithelial atrophy (prostatitis) were observed in 12% of young beagles (Glaister, 1986). Atrophy was reported in the absence of inflammation in 7 (2%) of 326 males by Hottendorf and Hirth (1974). Focal cystic hyperplasia was reported in 15 (5%) of 326 males by Hottendorf and Hirth (1974). In the immature prostate, there is no prostatic secretion (lumens are small) and glandular epithelia are small and flat (Sato et al., 2012).

Prepuce

Mild balanoposthitis (inflammation of the glans penis and prepuce) is common and may result in slight mucopurulent preputial discharge.

Ovary

Occasionally, clear cysts may be found in the vicinity of the ovaries of young adult beagles. The cysts have one of several origins (McEntree, 1990). The finding of parovarian cyst is not a specific diagnosis. Parovarian cysts were observed grossly in the ovaries of 2 (0.4%) of 499 dogs (Hottendorf and Hirth, 1974). Ovarian changes listed by Sato et al. (2012) include polyovular

follicles characterized by multiple ova present in one ovarian follicle, hyperplasia of the rete ovarii embryonic remnants, and mineralization of necrotic oocytes that accompany follicular atresia.

Uterus

Distention of the uterus was observed in 11% of young beagles (Glaister, 1986). Myometrial cysts have been reported in the uterus with one affected animal in 499 females (Hottendorf and Hirth, 1974). An occasional finding suggestive of pseudopregnancy is cystic epithelial proliferation of the endometrium separated into two layers in a placenta-like arrangement (Sato et al., 2012). Inflammation of the uterus or endometritis caused by *B. canis* infections can result in abortion during the third trimester of pregnancy (Jubb et al., 1992).

Neoplastic Findings, Spontaneous

Kidney

Spontaneous tumors are rare in the kidney of young dogs. A renal carcinoma was reported in one of a thousand laboratory dogs (Hottendorf and Hirth, 1974). A benign mixed mesenchymal tumor was detected in the kidney of an 11-month-old beagle (Robison et al., 1997).

Nervous System

Anatomy and Histology

The nervous system is the chief coordinating system of the body. The body's other major coordinating system, the endocrine system, is controlled by the nervous system and a major part of it (the hypothalamus) is also a major part of the nervous system. The overall function of the nervous system is to produce the proper reaction of the organism to changes in both the external and internal environment (Jenkins, 1978). This function depends directly on the neuron. Neurons, along with supportive cells (neuroglia), make up the nervous system. The nervous system is divided into the central nervous system (CNS) and the peripheral nervous system (PNS), more for discussion purposes than on the basis of structure or function. The CNS consists of the brain and spinal cord (neuraxis). The PNS consists of cranial and spinal nerves, sensory and motor ganglia, sensory and motor nerve endings, and the automatic nervous system.

The autonomic nervous system is that part of the nervous system concerned with motor innervation of smooth muscle, cardiac muscle, and glands. On anatomical, pharmacological, and functional bases, the autonomic nervous system is divided into a sympathetic portion and a parasympathetic portion. The general function of the sympathetic portion is to prepare the body for a state of emergency. The general function of the parasympathetic portion is to restore the body to a normal state of quiescence. The hypothalamus is the key to autonomic regulation. The rostral hypothalamic area controls the parasympathetic function and the caudal hypothalamic area controls the sympathetic function.

Divisions of the Nervous System: Structure and Function

All divisions of the nervous system are structurally and functionally connected. The PNS transmits impulses toward the CNS by way of sensory (afferent) neurons and away from the CNS by way of motor (efferent) neurons. The typical spinal nerve and the majority of cranial nerves have both types of functional neurons and are referred to as mixed nerves. A typical peripheral nerve consists of an outer connective tissue sheath (epineurium) enclosing bundles (fasciculi) of nerve fibers.

Each fasciculus is surrounded by its own connective tissue sheath (perineurium) and is made up of individual nerve fibers (microscopic in size) surrounded by their connective tissue sheaths (endoneurium). Individual nerve fibers consist of an axon and an enclosing sheath formed by lemmocytes (Schwann cells). The axon is the elongated cytoplasmic extension (process) of the nerve cell. The surface membrane of the axon is the axolemma. Most peripheral axons are surrounded by an insulating sheath of lipoprotein (myelin). Myelin is the nonnucleated plasma membrane of the Schwann cell. The nucleated portion of the Schwann cell is the neurilemma (regeneration of axons is possible in Wallerian degeneration owing to survival of the nucleated neurilemma). The terms "41 axolemma" and "neurilemma" are not synonymous, and neither "neurilemma" nor "myelin" is synonymous with the total "Schwann cell." The terms "axon" and "axis cylinder" are synonymous. Unfortunately, the total nerve fiber is also referred to as the axon.

Nerves of the CNS

The nerves attached to the brain are referred to as cranial nerves. There are 12 pairs, and except for the first pair (olfactory), all are attached to the brain stem. Nerves attached to the spinal cord are referred to as spinal nerves. There are usually 36 pairs of spinal nerves in the dog, derived from 36 spinal cord segments: 8 cervical, 13 thoracic, 7 lumbar, 3 sacral, and 5 coccygeal. With the exception of the first cervical nerve, all spinal nerves pass through intervertebral foramina. Because the spinal cord is shorter than the spinal column (in the embryo, the vertebral column grows more rapidly than the spinal cord, resulting in a relative caudal migration of vertebrae), there is not a one-to-one relationship between the spinal cord segments and the vertebrae. As a result, spinal nerves generally exit caudal to their segment of origin and vertebrae of one region may contain spinal cord segments of another region. The actual spinal cord segment/vertebral body correlations must be in mind when spinal cord segments are chosen for histological examination. The nerve distribution to the fore- and hindlimbs allows the spinal cord to be divided into functional units. The location of the units within vertebrae is shown in Table 8.14 (Bailey and Morgan, 1983).

The CNS is protected, supported, and nourished by three sheetlike connective tissue coverings called the meninges (singular: meninx). The outermost (and toughest) meninx is the dura mater. The dura mater of the cranial cavity serves a dual function: meningeal covering for the brain and endosteal (or periosteal) lining for the bones of the calvarium. The spinal dura mater is separated from the periosteum of the vertebrae by the epidural cavity. Clinical neurological signs help to localize precisely where in the brain, spinal cord, or PNS the problem may be. Based on these (and clinical laboratory) findings, the pathologist may modify the postmortem procedures as needed to assure that all appropriate areas are examined and all appropriate tissues are collected.

To minimize the handling of the brain, the head should be removed from the body. First, reflect the skin from the head and neck, sever neck muscles at their attachment to the posterior aspect of

Table 8.14   Vertebrae and Spinal Cord Segments

Cord Segment	Nerve/Tract Distribution	Vertebrae
Cervical, C1–C5	Nerve fiber tracts ascending from forelimbs and hindlimbs and descending from brain	C1–C4
Cervicothoracic, C6–T1/T2	Origin of nerves to forelimbs	C5–C7
Thoracolumbar, T2–L3	Nerve fiber tracts ascending from hindlimbs and descending from brain	T2–L3
Caudal lumbar, L4–L7	Origin of nerves to hindlimbs except S, contribution to the sciatic nerve	L3–L4
Sacral, S1–S3	Origin of nerves to the anus, urinary bladder, and perineum. S, contribution to sciatic nerve	L5
Coccygeal, C1–C5	Origin of nerves to tail	L5 caudally

the skull, and cut completely through the spinal cord at the atlanto-occipital articulation. The cut is made by passing a thin narrow blade through the dorsal atlanto-occipital membrane into the cisterna magna and then through the spinal cord. As the cisterna magna is entered, a quick appraisal of the CSF should be made. Expose the calvarium by removing the temporalis muscles. Remove the head by completing the disarticulation of the atlanto-occipital joint. (The disarticulation can be done quickly, if the prosector is methodical in cutting the joint capsule and the various ligaments.) Three cuts are made through the calvarium. One cut is made transversely through the frontal bone at the anterior limit of the cranial cavity slightly rostral to the zygomatic processes. The incision includes the frontal sinuses. Two identical cuts are made on each side of the calvarium, just dorsal to the widest part of the brain case. The lateral cuts connect the transverse cut with the foramen magnum. The foramen magnum should be entered at its widest point slightly dorsal to the occipital condyles. The cuts through the calvarium require practice and a sense as to when the saw is about to leave bone and enter the brain. Pull the calvarium upward and backward. Generally, the dura mater will strip from the calvarium and remain with the brain.

After examining the dura mater, cut it (and the arachnoid) parallel to the bone incisions. Two large folds of the dura mater will also need to be freed from their insertions between parts of the brain. The falx cerebri is the midsagittal fold between the cerebral hemispheres. The tentorium cerebelli is the transverse fold between the occipital poles of the cerebral hemispheres and the cerebellum. Failure to completely remove these folds will interfere with removal of the brain. The brain is extremely fragile and should be removed from the skull mostly by gravitational force and a little gentle traction. This is accomplished by holding the head upside down in one hand and freeing the brain by severing the cranial nerves, posteriorly rostrally, as they come to view. In the process, the brain gradually falls into the palm of the supporting hand. The hypophyseal stalk (infundibulum) is severed as soon as it is seen so that the hypophysis remains intact in the sella turcia. The optic nerves are cut and as much of the olfactory lobes are removed as possible. Examine the ventral surface of the brain as it lies in the palm of the hand.

Place the brain in a weighing boat and examine the dorsal surface. The brain need not be grasped, but may be slid from weighing boat to weighing boat or weighing boat to fixative. The removal of the hypophysis has been described in the endocrine system. The inner surface of the calvarium and ventral surface of the cranial cavity should be examined. The entire spinal cord, or segments of it, may be removed by either a ventral or dorsal approach. Before attempting to remove a segment, always completely transect the cord cranially and caudally to prevent stretching and twisting of the spinal cord as the spinal column is manipulated. The spinal cord should be removed by grasping the dura mater. If the entire spinal cord is to be collected, it should be fixed in a fully extended position in an adequately long container. Cervical and thoracolumbar segments of the spinal cord are easily removed from vertebrae C1-C4 and T2-L4, respectively. These segments sample ascending and descending tracts of all the limbs and the nerves of origination to the hindlimbs. Traditionally, a portion of a sciatic nerve is collected as the representative sampling of the PNS. The sciatic nerve can be removed with minimal trauma and is large enough to provide adequate samples (5 cm or longer). The sciatic nerve contains both afferent and efferent nerve fibers (mixed nerve). If a sensory nerve (afferent fibers) is required, a branch of the sciatic nerve, the caudal (lateral) cutaneous sural nerve, may be taken. The caudal cutaneous sural nerve has been studied in healthy adult beagles and morphometric and electrophysiological data exist for it (and the ulnar and saphenous nerves) (Illanes et al., 1988). Artifacts in nervous tissue can be as troublesome as artifacts in muscle tissue. The makeup of nervous tissue makes it unusually sensitive to autolysis, rough handling, and the chemical effects of fixative. Primary fixation by whole body perfusion with buffered glutaraldehyde addresses most of the problems and can be performed as successfully in the dog as in smaller laboratory animals. However, whole body perfusion has practical application only in selected studies, leaving the problem of tissue artifacts in the brain, spinal cord, and peripheral nerves to be dealt with on a daily basis in routine studies. With the brain, the choice is between

the artifacts of handling and distortion from slicing a brain in an unfixed state and the artifacts of autolysis in brains fixed by immersion. The choice is usually to fix the brain in toto and examine for gross internal lesions when the brain is sectioned for processing. Full transverse (coronal) sections are preferred for microscopic examination. Cross and longitudinal sections of the spinal cord should be prepared. Specimens of peripheral nerves should be at least 5 cm in length and removed with great care. Peripheral nerves should be fixed in a gently stretched state on cardboard or corkboard. Ten percent neutral buffered formalin is the traditional fixative for nervous tissue. Brains should be fixed in large volumes of fixative, which should be changed frequently. The investigator may wish to treat peripheral nerve specimens as surgical specimens and follow a nerve biopsy protocol (Asbury and Johnson, 1978), which provides for plastic and/or paraffin embedding; routine, thick or thin sections; special stains; and nerve fiber microdissection (nerve teasing, teased specimen). Routine sections of peripheral nerves should always include transverse and longitudinal sections. H&E is the most universally used stain; however, it does not stain specific nerve components and special stains are required for myelin, Nissl substance, neurofibrils, neuroglia, etc. The writers fully agree with Dr. Jenkins (1978): "Neurostaining is a special technique which ideally should be reserved only for the experienced histotechnician who has the time and interest to devote to the subject." Microscopists who are unfamiliar with the artifacts commonly encountered in peripheral nerves are referred to Asbury and Johnson (1978). Readers interested in contemporary neuropathological methods in toxicology are referred to Spencer and Bischoff (1982).

Pathology

Hydrocephalus is a common nervous system finding in laboratory dogs. It was observed grossly in 14 (1%) of 1000 dogs on 39 studies (Hottendorf and Hirth, 1974). Hydrocephalus (ventricular dilatation) associated with a spongelike alteration in the surrounding brain tissue was observed by Oghiso et al. (1982). Several beagles with hydrocephalus were reported by Fritz et al. (1967).

Hydrocephalus is characterized by abnormal accumulations of CSF. It is usually manifested as dilatation of one or more ventricles of the brain (internal hydrocephalus), but ventricles may be unaffected and it is the subarachnoid space that is dilated with the excess CSF (external hydrocephalus). If excess CSF is present in both locations, the condition is referred to as communicating hydrocephalus (Sullivan, 1985). While hydrocephalus appears to be fairly common in laboratory beagles, it is likely that minor degrees of ventricular dilatation are unrecognized and the real incidence of the condition is greater than reported. Lateral ventricles of surprising size (some with thinning or rupture of the septum pellucidum) are found incidentally while trimming brains of dogs that showed no neurological signs. At necropsy, the ventral surface of the brain should be closely examined, because the piriform area will dimple under slight pressure even in mild hydrocephalus. Hydrocephalus in laboratory beagles is probably a congenital condition.

Inflammation of the nervous system was also frequently reported. Chronic focal meningitis was observed in brains from 44 (7%) of 630 dogs on 33 studies and focal encephalitis in 5 (0.8%) of these 630 dogs (Hottendorf and Hirth, 1974). Focal inflammatory cell infiltration (lymphocytes and plasma cells) is occasionally found in the interstitium of the choroid plexus (Sato et al., 2012). This should be distinguished from extramedullary hematopoiesis, which also affects the choroid plexus but includes hematopoietic cells of granulocytic series with rare megakaryocytes. Focal myelitis was observed in spinal cords from 2 (0.3%) of 647 dogs (Hottendorf and Hirth, 1974). Toxocar granulomas in the brain and spinal cord (cauda equina) were reported by Barron and Saunders (1966). Granulomatous meningoencephalitis is an idiopathic inflammatory condition of the CNS of dogs characterized by focal or disseminated granulomatous lesions and perivascular cuffing predominantly in the white matter of the brain and spinal cord and nonsuppurative meningitis (Braund, 1985; O'Neill et al., 2005). Granulomatous leptomeningitis characterized

by noncaseating granulomas in the leptomeninges and slight scattered perivascular cuffing in the gray and white matter throughout the CNS was reported in seven laboratory beagle dogs by Maeda et al. (1993). Maeda et al. (1993) also reported the presence of *Escherichia coli* antigen in the cytoplasm of macrophages in these lesions and considered granulomatous leptomeningitis, distinct from granulomatous meningoencephalitis. Ueda et al. (2004) reported nonsuppurative meningoencephalitis with disseminated foci of perivascular mononuclear cell cuffing in a 15-month-old male beagle dog. Perivascular cuffing of lymphocytes and mononuclear cells is seen occasionally as an incidental finding in laboratory beagle dogs (Ueda et al. 2004). Juvenile polyarteritis in beagle dogs is reported to affect small- and medium-sized arteries of the cervical spinal meninges (Snyder et al., 1995).

Hemorrhage involving the brain was reported by Pick and Eubanks (1965). They did not elaborate on the location or extent of the hemorrhage. A rare hamartomatous lesion in the brain was reported by Sato et al. (2012) and thought to possibly represent ectopic tissue. The lesion consisted of choroid plexus components mixed with adipose tissue, collagen, and small blood vessels. A senile change in the spinal cord is mineralization of vessel walls in the spinal nerve roots (Sato et al., 2012). Mineralization and, rarely, fibrous thickening also occur in the meninges of the brain.

Hottendorf and Hirth (1974) observed, but did not report as lesions, small subependymal collections of glial cells usually seen around the anterior parts of the lateral ventricles. Such foci have been described as glial rests. Spherical, eosinophilic granular structures have been observed in the medulla oblongata, pons, or anterior cervical cord. They seem to be most common in the gracilis tract and nucleus. Newberne et al. (1960) and Innes and Saunders (1962) believe the structures are degenerating axis cylinders. They apparently have no neurological or pathological significance. Spontaneous degenerative lesions of the peripheral nerves occur in the beagle as in other laboratory animals. While commonly considered an aging change, occasionally "digestion chambers" and "myelin bubbles" are seen in the sciatic nerves of young beagles.

During examination of peripheral nerves, cylindrical, loosely textured, whorled, cell-sparse fine fibrous structures are occasionally observed within the nerves. These are Renaut bodies and were well known to histologists of the late nineteenth century, but they have gradually been forgotten (Asbury, 1973; Asbury and Johnson, 1978). Renaut bodies are particularly well developed in the horse and donkey but are less conspicuous in the dog and humans. They are thought to protect the peripheral nerve fibers from pressure damage (Sato et al., 2012). It is important that they be recognized as normal structures and not misdiagnosed as nerve infarction or necrotizing angiopathic neuropathy, as has been done in the past (Asbury, 1973).

Eye and Ear

Anatomy and Histology

Eye

The eye is the organ of vision. It is composed of the eyeball (globe), the optic nerve, and accessory structures including the eyelids, conjunctiva, lacrimal apparatus, and ocular muscles. The canine eye is relatively large for the size of the animal. The human eye is approximately 2.5 cm in diameter (Kuwabara and Cogan, 1977); the eye of an adult beagle is approximately 2.2 cm in diameter (Andersen, 1970). The canine eye is also placed well forward in the head, giving the dog a large field of binocular vision. The eyeball is roughly spherical in shape, with the rostral curvature of the cornea making the anterioposterior diameter the greatest diameter of the eye. To facilitate description and orientation of the eye, special designations as to side, direction, and position are used. Oculus dexter designates the right eye, oculus sinister designates the left eye, and oculus unitus designates both eyes. The side of the globe nearest the nose is the nasal, or medial, side; the

opposite side is the temporal, or lateral, side. The dorsal side is superior; the ventral side is inferior. The corneal pole is distal, or anterior; the cerebral pole is proximal, or posterior. The line connecting the two poles is the anatomical or optic axis. The equator is the greatest expansion of the eyeball perpendicular to the anatomical axis. A horizontal plane through the poles divides the eyeball into an upper half and a lower half. A vertical plane through the poles divides the eyeball into a nasal half and a temporal half.

Eyeball

The eyeball consists of an inner coat, or tunic, of neural light-sensitive tissue (retina) held in shape by surrounding coats that protect it (corneoscleral coat, outer fibrous coat) and nourish it (uvea, middle vascular coat). The outer fibrous coat is subdivided into a larger, tough, white posterior portion (sclera) that covers about 0.75 of the globe and a smaller, transparent anterior portion (cornea). The transition from the opaque sclera to the transparent cornea is comparatively abrupt and occurs at the corneoscleral (sclerocorneal) junction, or limbus. The limbal area of the sclera is pigmented laterally and medially but not dorsally or ventrally.

Sclera

The thickness of the sclera varies. At the equator, it is so thin as to be semitransparent and the dark color of the uvea shows through. It is thick at the ciliary region where extraocular muscles insert and around the optic nerve.

Cornea. The cornea is transparent, colorless, and nonvascular; however, it possesses dense nerve fiber plexuses and is highly sensitive. Opposite to what exists in humans, the cornea of the dog is thicker in the center than at the periphery (Getty, 1967). The transparency of the cornea is due to a nonkeratinized and nonpigmented surface epithelium, lack of blood vessels and lymphatics, cell-poor stroma composed of thin collagen fibrils arranged in orderly lamellae, and a sodium–potassium pump in the cell membrane of the corneal endothelium that maintains a high degree of stromal dehydration (Wilcock, 1985). The composition of the cornea is notably uniform and consists of six or seven layers (six in the dog). The outermost and often overlooked layer is the tear film (not seen in histological sections). The outer stratified squamous epithelium is a continuum of the conjunctival epithelium. The basement membrane of the epithelium is the third layer. According to Dellmann (1976c), the dog does not have an anterior limiting membrane (Bowman's membrane). However, Prince et al. (1960) indicate that an anterior limiting membrane is present, but that it is extremely thin (1.5 vs. 30 mcm in humans).

Shively and Epling (1970), in their study of the fine structures of the beagle eye, found no consistent layer of randomly oriented collagen fibers that would constitute an anterior limiting membrane. The stroma constitutes the bulk of the cornea. Descemet's membrane is present (a required constituent, since it is the basement membrane of the innermost layer, the endothelium). The endothelial layer should probably be known as mesothelium based on its structural characteristics and its probable origin (Shively and Epling, 1970).

Uvea

The uvea is the highly pigmented and vascular coat of the eye. It consists of the iris, ciliary body, and choroid.

Iris

This is the most anterior portion of the uveal tract and is the diaphragm of the eye.

Ciliary Body

This is a ring of tissue that extends from the base of the iris to the neurosensory retina posteriorly. The main components of the ciliary body are the ciliary processes and the ciliary muscle.

The ciliary processes are actually linear folds that when seen from the rear appear as multiple radiating ridges. The ciliary processes are highly vascular and are thought to be the main sites for formation of the aqueous humor (an arrangement comparable to the formation of CSF by the choroid plexuses of the brain).

In accommodating for near vision, the ciliary muscle contracts, pulling the ciliary body forward, which allows the supporting fibers (zonule fibers) of the lens to relax, leading to relaxation and an anterioposterior thickening of the lens.

Choroid

The bulk of the uvea is formed by the choroid, which consists mainly of blood vessels and melanocytes. Externally, it blends with the sclera; internally, it is bounded by a basal lamina called Bruch's membrane; and dorsally (above the optic disc), it displays a peculiar structure known as the tapetum lucidum.

Tapetum

Many animals have one of two types of tapeta: tapetum lucidum fibrosum or tapetum lucidum cellulosum. The dog has a tapetum lucidum cellulosum. The tapetum lucidum is a light-reflecting layer (responsible for the luster of eyes) that is situated in the dorsal half of the choroid. The tapetum is triangular to semicircular in shape, extending about halfway to the periphery of the choroid, and has a horizontal base that just contacts the top of the optic disc. Although the tapetum appears to be immediately adjacent to the pigment epithelium cells of the retina, there is a layer of capillaries (choriocapillaris) between the tapetum and Bruch's membrane. The tapetum is made up of flat cells varying in number from 9 to 10 layers centrally to 1 or 2 layers peripherally (Prince et al., 1960).

Retina

The innermost tunic of the eye is the retina. It is divided into a sensory portion (pars optica) that rests upon the choroid and a nonsensory portion that rests upon the ciliary body (pars ciliaris) and the posterior surface of the iris (pars iridica). The sensory and nonsensory portions join posterior to the ciliary body at a scalloped border known as the ora serrata. The sensory retina is firmly attached at the ora serrata and optic disc, but it is loosely attached over the choroid. Under normal circumstances, the pressure of the vitreous is sufficient to hold the retina in contact with the choroid.

The nonsensory retina consists of a simple layer of pigmented cuboidal epithelial cells that continues at the ora serrata to form the outermost of the 10 layers of the sensory retina. Not all of the pigment epithelium is pigmented; the cells overlying the tapetum lucidum are not. According to Kuwabara and Cogan (1977), two pigments may be found in the pigment epithelium: melanin and lipofuscin. The pigment epithelium interdigitates with the overlying photoreceptors. Among other functions, the epithelial cells engulf and degrade obsolete rod and cone cell segments. The accumulation of lipofuscin appears to be a result of this phagocytic activity. In humans, the quantity of lipofuscin increases with age and the number of melanin granules decreases (Kuwabara and Cogan, 1977).

The inner transparent layer of the sensory retina comprises nine layers, named from within outward: internal limiting membrane, nerve fiber layer, ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, external limiting membrane, and the

layer of rods and cones. The retinal receptors in the dog are predominantly rods. That feature (and others) indicates that the dog is predominantly a nocturnal animal (Prince et al., 1960). To the ophthalmologist, the visible portion of the posterior globe is the ocular fundus, which for descriptive purposes is commonly divided into a dorsal tapetal fundus and a ventral nontapetal fundus (tapetum nigrum). The optic disk is usually at the junction of the two. The optic disk (optic papilla) is the rounded, raised area where the optic nerve leaves the eye. Jenkins (1978) emphasizes the following important features relative to the optic nerve: since the optic nerve is a fiber tract of the brain, it has no neurilemmal sheath of Schwann and regeneration is not possible; it contains neuroglial elements; it has a meningeal investment but no epi-, peri-, or endoneurium; and the individual nerve fibers are all myelinated. Wilcock (1985) points out that, in contrast to most domestic species, the fibers in the optic papilla of the dog are myelinated. Since the optic nerve is in direct contact with the eye and brain via neurons and CSF, it may be affected by diseases of both the eye and brain.

Branches of the ciliary and internal ophthalmic arteries and veins lie just along the optic nerve. When they reach the eye, some plunge into the globe and others (medial and lateral long posterior ciliary arteries and veins) pass into the superficial layers of the sclera and are externally visible for a distance along the horizontal meridian of the eye. These vessels serve as landmarks in orienting the eye at trimming.

The transparent media of the eye include the cornea, aqueous humor, lens, and vitreous body (the retina, excluding the pigment epithelium is also transparent). The aqueous humor is the water-clear fluid contained in a cavity bounded anteriorly by the cornea and posteriorly by the lens. The space between the cornea and the anterior surface of the iris is the anterior chamber and the space between the posterior surface of the iris and the lens is the posterior chamber. Aqueous humor is produced in the posterior chamber and drains from the anterior chamber into a meshwork of channels located at the junction of the cornea, sclera, and iris (iris angle, filtration angle).

The lens is composed entirely of epithelial cells whose basement membrane is the thick outermost capsule of the lens. The bulk of the lens is formed by layers of epithelial processes that cannot be shed, but are compacted with age to the center of the lens. The adult lens depends entirely upon the aqueous humor for delivery of nutrients and removal of wastes. The vitreous body is the transparent gel that fills the inner portion of the eyeball between the lens and the retina. The vitreous shows considerable shrinkage with most fixatives (over 99% of the vitreous is water) and its normal, in-life distribution is not apparent in microscopic sections.

Third eyelid. The dog has a third eyelid, or nictitating membrane, located at the medial angle of the palpebral aperture (medial canthus, nasal canthus). A T-shaped hyaline cartilage forms the skeleton of the third eyelid. The cross of the "T" supports the free margin of the lid. The lower shaft of the "T" is surrounded by a mixed lacrimal gland (superficial gland of the third eyelid, nictitans gland). According to Getty (1967), the dog does not have a Harder's gland (deep gland of the third eyelid). The inner conjunctival covering (bulbar conjunctiva) contains numerous lymphoid nodules.

Ear

The ear is the organ of hearing and equilibrium. It is composed of three connected divisions, each of which is referred to as an ear: external (outer) ear, middle ear, and internal (inner ear). The inner ear is the organ for both hearing and equilibrium. The external and middle ears are sound-collecting and sound-conducting apparatuses (Getty, 1967).

External Ear

This consists of the pinna (auricle) and the external auditory meatus (ear canal). The pinna is composed of a sheet of elastic cartilage (auricular cartilage) covered by skin on both sides. The auricular cartilage is pierced by many foramina that permit the passage of blood vessels. Histological sections

that include foramina give the impression that the cartilaginous sheet is not continuous. The function of the pinna is to direct air vibrations into the funnel-like ear canal to the tympanic membrane (ear drum). The tympanic membrane is the partition between the external ear and the middle ear.

Middle Ear

This consists of the tympanic cavity, the auditory tube, and a chain of three small bones (auditory ossicles) that connect the tympanic membrane to the oval window of the inner ear. The tympanic cavity is air filled and communicates with the nasal pharynx by means of the auditory tube (eustachian tube).

Inner Ear

This is contained within the temporal bone and consists of the membranous labyrinth containing the organs of hearing and equilibrium and the bony labyrinth surrounding the membranous labyrinth. The inner ear is fluid filled. The bony labyrinth, which lodges the membranous labyrinth, contains a fluid, perilymph, and communicates with the cerebrospinal space by way of the vestibular aqueduct. The membranous labyrinth is filled with a fluid called endolymph.

Necropsy and Laboratory Techniques

Eye

Many lesions of the eyelids, conjunctiva, and cornea will be clinically apparent; however, most lesions within the globe will be hidden to the pathologist without the assistance of an ophthalmoscopic examination. Ideally, the pathologist will have the notes, drawings, or photographs of an ophthalmologist to direct him or her to lesions within the eye. Minute lesions can be difficult to find without directions and lesions involving the lens may be obscured or lost because of the difficulty in obtaining complete, artifact-free sections of the lens. Autolytic changes can be detected in the canine retina within 5 min of death (Saunders and Rubin, 1975). Unless there are compelling reasons to do otherwise, the eyes should be the first organs removed at necropsy and both eyes should be in fixative well within the first 5 min of the necropsy. To meet the 5 min deadline, all preliminary procedures (weighing, external examination, clipping of hair) should be done prior to exsanguination. Good exsanguination limits the degree of hemorrhage into the orbit during dissection. The enucleation can be done by a single prosector, but the assistance of a second person is required to have both eyes removed, cleaned, and in fixative within the 5 min time frame.

A single incision through the lateral palpebral angle will free the upper and lower eyelids sufficiently to give access to the eye. With an assistant stabilizing the head and stretching the skin to spread the eyelids, the prosector grasps a lateral fold of palpebral conjunctiva with tissue forceps and with curved blunt scissors (a writer, RWT, prefers Metzenbaum scissors) makes an initial incision in the conjunctiva behind the forceps. With gentle traction and well-directed cuts with the scissors, the prosector severs the conjunctiva and extraocular muscles on the lateral, dorsal, and ventral sides of the eye, working toward the cerebral pole where the optic nerve and remaining muscles are cut. The eye is pulled gently forward and remaining extraocular tissue is cut, including the third eyelid. The position of the forceps never need be changed. The globe is placed on a wet sponge to cushion it and the extraocular muscles, fat, and fascia are gently removed close to the sclera, using the convex surface of the scissors. Although the left- and right-side identity of the eyes may be made using anatomical landmarks, it is easier to maintain the identity of the eyes by placing the globes in identified containers or to tie a piece of black thread to a bit of loose extraocular tissue oil one of the eyes. The eyes are gently lowered by the thread or forceps into the fixative. At least 5 mm of

optic nerve should be left attached to the eye. With proper cleaning, the eye will sink in fixative. The thorough cleaning is required to permit rapid penetration of fixative and to prevent retinal detachment because of the pressure exerted by contracting muscles. For the same reason, the globe should not be grasped by the fingers. Usually, eyes are not weighed. Measuring the diameters of unfixed globes is time consuming and increases the likelihood of retinal detachment. The volume of an eye is easily determined by displacement. Choose a graduated cylinder with an inside diameter just great enough to accept an eye. Partially fill the cylinder with fixative, read the volume, lower the eye into the fixative, and read the new volume. The difference in the volume readings is the volume of the eye. Using an appropriate cylinder, the volume of a beagle eye can be measured to 0.2 mL. After measuring, the eye is poured into the primary container of fixative.

There appears to be no ideal fixative for the eye. Ten percent neutral buffered formalin generally gives good results for the cornea and lens but poor results for the retina. Rapidly penetrating fixatives such as Zenker's, Helly's, and Bouin's are generally good for the retina but require strict attention to a fixation schedule to avoid overfixation. Many additional fixatives including ethanol have been used. A writer (RWT) has had good results with 2.5% phosphate buffered glutaraldehyde. Dog eyes placed in this fixative may be "cut in" in 1.5–2.0 hours, allowing a gross examination, while the tissues retain much of their original color and the lens is still relatively clear. Fixation is complete in about 72 hours. Davidson's fixative (Humason, 1972) is a simple, easy-to-use fixative that gives good results. Unless there is good reason to do otherwise, the canine eye should be trimmed perpendicular to the horizontal meridian (posterior ciliary vessels) to obtain a midsagittal block that includes the tapetal and nontapetal fundus and optic nerve. A complete sampling of the eye would include a transverse section of the optic nerve. Preparing top-quality eye sections requires special skill and (as with neurohistological technique) should be reserved for the experienced histotechnician with the time and interest to devote to the subject.

Ear

The middle and inner ears are not routinely examined in toxicology studies; however, it is not difficult to collect and prepare sections that include the tympanic membrane and middle and inner ears. The external ear is removed close to the tympanic membrane. After removal of the brain and disarticulation of the mandible, two transverse cuts are made through the base of the skull just anterior and posterior to the external acoustic meatus. One of these cuts will usually just enter the tympanic cavity of the middle ear. After ample fixation and decalcification (the temporal bone is very dense), the specimens may be trimmed and processed in a routine manner.

Pathology

Only Glaister (1986) reported ocular disease, but his observations are limited to sores and conjunctivitis without describing these lesions. The conjunctivitis was associated with sawdust bedding and dust in 4% of young beagles. Oghiso et al. (1982) described lymphoid cell infiltration of the third eyelid and gland of the third eyelid. Hottendorf and Hirth (1974) examined both eyes in 85% of their studies but reported no ocular pathology in 630 beagles. Rubin and Saunders (1965) reported *Toxocara* granuloma in the retina or choroid in three young beagles. Focal lymphocytic infiltration in the subepithelium of the conjunctiva is a common background finding. This change is also commonly found in the lacrimal glands where it may be accompanied by focal fibrosis and acinar atrophy (Sato et al., 2012).

Heywood et al. (1976) followed ocular changes in 86 laboratory beagles from 6 months to 8 years of age, at which time the animals were necropsied. Prominent posterior lens sutures were the only ophthalmological change seen in beagles under 3 years of age. From 3 to 8 years of age, ophthalmological changes included corneal opacities, asteroid bodies, and tapetal pigmentation. For the lens,

they reported prominent posterior sutures, nuclear opacities, and anterior, posterior, and peripheral capsular opacities. Histological findings at 8 years of age included keratitis, cystoid degeneration of the retina, thinning, and absence of tapetal cells, pigment cells in rod and cone layer, scarring of the retina, and calcified body between the pigment epithelial layer and choroid.

Schiavo and Field (1974) examined 532 beagles of various ages ophthalmoscopically and biomicroscopically and reported the following in dogs between 6 and 12 months of age: superficial keratitis in 35 (6.6%), deep keratitis, posterior polar opacities, posterior cortical opacities in 54 (10.1%), prominent posterior lens sutures, lenticular sheen, vacuoles in lens cortex, persistent hyaloid vessel remnants, zones of discontinuity (lens), vitreous floaters/filaments, atapetal fundi, tigroid fundi, tapetal aberrations/pigment clumps, peripapillary reflectivity, and increased tapetal reflectivity. Lenticular sheen is a yellowish reflection from the lens media and is generally expected as a senile change. It was observed in 120 (22.6%). The hyaloid artery is responsible for the development of the vitreous and for nourishment of the fetal lens. Normally, the vessel regresses shortly after birth but sometimes remains to be seen in the adult. The incidence of persistent hyaloid vessels was 142 (26.7%). Zones of discontinuity reflect stratification of lens fibers and are thought of as a presenile change. They occurred in 32 (6%). Vitreous floaters are asteroid bodies (small calcium bodies). Vitreous filaments are fibrous strands from vitreous hemorrhage or remnants of the posterior vascular capsule. The incidence of vitreous floaters and filaments was 53 (10%). Tigroid fundi describe the appearance of choroidal vessels through areas of nonpigmented pigment epithelium. Tapetal aberrations include areas of hyperreflectivity and old hemorrhage or scars. These aberrations occurred in 25 (4.7%). Bellhorn (1974) reports similar ocular findings in 8–10-month-old beagles plus prolapse of the third eyelid.

The abundance of ophthalmoscopic observations and the paucity of histological findings emphasize the comparative sensitivities of the two modes of examination. The discrepancy is easily understood for the ophthalmologist has the entire living cornea, iris, lens, and fundus to examine. On the other hand, the pathologist is limited to one or two thin sections of an organ that is both difficult to fix and difficult to section.

Peripheral retinal cystoid degeneration describes single or multiple microcysts within the retina at or near the ora serrata. This is a common change in older beagles, but it also occurs in young animals. At 8 years, the incidence rate was 85%. The lesion has no apparent functional significance. Focal retinal dysplasia manifested as retinal folds, retinal rosettes, focal absence of retinal cells, and blending of nuclear layers is not an infrequent finding. Retinal folding may be the result of retinal dysplasia or developmental damage of the retina or failures of optic fissure closure (Sato et al., 2012). Protrusion of the third eyelid may be seen in young beagles. Histological findings include inflammation of the bulbar conjunctiva and stroma of the superficial gland. The ducts of the gland may be dilated and contain leukocytes. The conjunctival lymphoid tissue is usually hyperplastic.

There is no mention of ear pathology in the articles reviewed. Cutaneous lesions of the external ear were discussed with the integumentary system.

METABOLISM

Christopher J. Kemper

Among the nonrodent species, the dog has been the best characterized with regard to xenobiotic metabolism. This is probably because the dog has been extensively used in biomedical research for more than 100 years. Its relatively large size also provides the advantage of allowing repeat serial sampling of large amounts of biological fluids for time-course analyses. Data on the dog are a staple in numerous review articles and book chapters published on species comparisons elsewhere (see Chapter 1). The focus here will not only be on species differences but also on other items (e.g., inducibility) that have not necessarily been covered in detail elsewhere. Insofar as the beagle

dog is the most common breed and the most common nonrodent model used in toxicity testing, this chapter will focus on data from beagles and present data from other breeds where available.

At the onset, it should be noted that dog is generally believed to have a higher intestinal pH than man leading to some differences in oral absorption of more water soluble drugs (Dressman, 1986; Lui et al., 1986). Although otherwise similar motility patterns and pH profiles prevail in the two species for the most part, there are some differences that could affect the time profile and extent of drug absorption. These include slower gastric emptying in the fed state, faster small intestine transit, and higher and more variable intestinal pH in dogs compared with humans. An attempt is made to identify drug and dosage-form properties that would lead to differences in a drug absorption in the two species, e.g., drug physicochemical properties, dosage-form size, and pH dependency of dosage-form release characteristics.

As reported by Gregus et al. (1983), the dog has a somewhat smaller liver to body weight ratio than the rat (2.3% vs. 4.0%) and somewhat less microsomal protein (20.5 vs. 25.7 mg/g liver). Identified P450 CYP isozymes are listed in Table 8.15. Some of the more common parameters of hepatic xenobiotic metabolism in the dog are summarized in Table 8.16. Concentrations of cytochrome P-450 range from about 0.30 to 0.80 nmol/mg protein. Using HPLC and other techniques, Amacher and Smith (1987) characterized the cytochrome P-450 isozymic "fingerprint" of naive beagle dogs. Their results suggested that cytochrome P-450 exists as three distinct isozymic groups in female dogs and two main groups and two to three subgroups in male dogs. Further, they identified sex-related differences in chromatographic behavior between the major isozymic groups. This is an interesting finding because few, if any, sex-related differences in the activity of the microsomal mixed function oxidase (MMFO) have been identified in dogs. If Table 8.15 is compared to similar endpoints for man or other model species (such a table being presented in other chapters), it is readily seen that the dog is frequently not a good metabolic model for man and is poorly comparable to the rat and mouse.

McKillop (1985), using SDS-PAGE techniques, identified three major cytochrome P-450 isozymes in uninduced adult male dogs. Phenobarbital increased the levels of two of these major isozymes but primarily caused an increase in another (fourth) isozyme. β-Naphthaflavone caused increases in yet another three other isozymes that are only present in very small amounts in naive animals. Hence, as a generality, the beagle dog has at least seven isozymic forms of cytochrome P-450, including a cytochrome P-448 with some homology with that of the rat, but which are differentiated on the basis of molecular weight, substrate specificity, and responses to inducers.

In general, the dog has less ability than the rat to form hydroxylated aromatic metabolites via the MMFO. For example, in the metabolism of amphetamine, the dog produces very little 4-hydroxyamphetamine (as reviewed by Williams, 1971). Cook et al. (1982) reported that the dog, as opposed to the rat, produces no phenoxyl metabolites of disopyramide. Aniline-hydroxylating activity in

Table 8.15   Identified P450 CYP Isoforms Active in the Dog

CYP	1A1	(Low km—Shou et al., 2003)
CYP	1A2	
CYP	2A6	(Chauret et al., 1997)
CYP	1B11	
CYP	2C19	(Chauret et al., 1997)
CYP	2C21	(Low km—Shou et al., 2003)
CYP	2C41	
CYP	2D6	(Chauret et al., 1997)
CYP	2D15	(Low km—Shou et al., 2003)
CYP	2E11	
CYP	3A4	(Chauret et al., 1997)
CYP	3A12	(High km—Shou et al., 2003)
CYP	3A26	

Table 8.16 CYP-Specific Metabolic Activities in Beagle Dogs

Activity	Beagle Dog	References
7-Ethoxyresorufin O-dealkylation	1A1/2	Edwards et al. (1994) and Kastner and Neubert (1994)
7-Methoxyresorufin O-dealkylation		
Caffeine 3-demethylation		
Benzphetamine N-demethylation		
7-Benzoxyresorufin O-dealkylation	2B11	Ohmori et al. (1993)
7-Pentoxyresorufin O-dealkylation	2B11	Ohmori et al. (1993)
Coumarin 7-hydroxylation		
7-Ethoxy-4-trifluoromethylcoumarin deethylation		
Ethoxycoumarin O-dealkylation		
Tolbutamide methyl-hydroxylation		
Chlorzoxazone 6-hydroxylation		
Mephenytoin N-demethylation	R enantiomers twice as fast as S	Yasumaori et al. (1993)
4-Nitrophenol hydroxylation		
N-Nitrosodimethylamine N-demethylation		
Androstenedione 15α-hydroxylation		
Androstenedione 16α/β-hydroxylation	2B	Igarashi et al. (1997)
Dextromethorphan O-demethylation	2D15	Schulz et al. (1998)
Dextromethorphan N-demethylation		
Testosterone → androstenedione*		
Testosterone 2α-hydroxylation		
Testosterone 2β-hydroxylation		
Testosterone 6β-hydroxylation	3A12	Igarashi et al. (1997)
Testosterone 7α-hydroxylation		
Testosterone 15α-hydroxylation		
Testosterone 15β-hydroxylation		
Testosterone 16α-hydroxylation	2B11, 2C21	Ohmori et al. (1993)
Testosterone 16β-hydroxylation	3A12, 2B11[46]	Ohmori et al. (1993)
Lauric acid 11-hydroxylation		
Lauric acid 12-hydroxylation		

dogs tends to be less than that of rats. Gregus and colleagues (1983) have reported benzo(a)pyrene-hydroxylating activity in the dog is much less than that of the rat. There are exceptions; for example, the dog has a much higher rate (15 times) of metabolism with 2,2′,4,4′,5,5-hexachlorobiphenyl than the rat (Duignan et al., 1987). Some care must be taken in interpreting data on aniline hydroxylase in the dog, as this species produces both ortho- and para-aminophenol from aniline at a ratio of 1/2 (p/o) (Williams, 1971). In the rat, this ratio is 6/1. As the common colorimetric method (formation of a quinolinidine complex with phenol) of determining aniline hydroxylation is specific for p-aminophenol, the activities reported for aniline hydroxylase in the dog are probably low. With many other typical substrates (dealkylation rather than aromatic hydroxylation), MMFO activity in the dog is often comparable to those of the rat. Microsomal aminopyrine metabolism, for example, is about the same in dogs as in rats (Lan et al., 1983). Gregus et al. (1983) reported that the rat had about twice the activity with benzamphetamine, but four times the activity than with ethylmorphine than dogs. In contrast, dogs have a higher baseline level than rats for 7-ethoxycoumarin (primarily a substrate for P-450-dependent MMFO) and 7-ethoxyresorufin (primarily a substrate for P-448-dependent MMFO) deethylating activities (Gregus et al., 1983; McKillop, 1985), but greater degrees of induction (with either phenobarbital or β-naphthaflavone) occur in the rat than in

the dog (McKillop, 1985). Thus, in comparing the rat and the dog (probably the two most common species in toxicity testing), one should not assume that the rat has the more rapid rates of MMFO activity but does have a more extensive range of activities.

Nelson et al. (1996) and Zuber et al. (2002) report that the dog CYP1A, 2E1, and 3A12 and 3A26 activities are not well correlated with those in humans. CYP2D15 is well correlated with human CYP2D6 activity. The dog is also the only mammalian species able to metabolize polycyclic aromatic hydrocarbons through its CYP2BN enzyme.

There is considerable evidence that the MMFO of rats has stereospecificity. For example, Heimark and Trager (1985) compared the microsomal metabolism of R and S warfarin. The overall rate of oxidation (total product) was much greater with the R enantiomer. There is some evidence for MMFO stereospecificity in the dog. Cook et al. (1982) examined the disposition of (R) and (S) disopyramide in vivo in dogs. The (R) enantiomer had significantly longer half-life than the (S). In addition, a much higher percentage of dose of S-disopyramide was excreted in the urine as the major metabolite. In general, one should expect stereoselective metabolism of racemic mixtures in the dog.

Dog 1A and 2E1 are somewhat different than humans and are not typically good models for humans. Only mammalian species were able to metabolize polycyclic aromatic hydrocarbons through CYP2B11 (Zuber et al., 2002).

Celecoxib, a cyclooxygenase–2 inhibitor, had its pharmacokinetics in dogs evaluated as part of its nonclinical development program prior to FDA approval. Celecoxib is extensively metabolized by dogs to a hydroxymethyl metabolite, with subsequent oxidation to the carboxylic acid analog. Of the major CYP enzymes, CYP 2D15 was extensively involved polymorphism of metabolism in dogs was probably due primarily to other CYP subfamilies than 2D (Paulson et al., 1999).

Higher urea synthesis of both fresh and thinned liver slices than those of rat, cynomolgus monkeys, rhesus monkeys, hamsters, minipigs, and rabbits. But lower testosterone metabolism than any of other species (Kanter et al., 1997).

MMFO is inducible in the dog. McKillop (1985) examined the inducing effect of phenobarbital and β-naphthaflavone in beagle dogs. Phenobarbital (in saline) was administered ip for 7 days, 20 mg/kg for 2 days, 10 mg/kg for 2 days, and 20 mg/kg for the final 3 days. β-Naphthaflavone in arachis oil was given ip for 6 or 7 days, 10 mg/kg. The phenobarbital treatment increased cytochrome P-450 by approximately 250%, whereas the β-naphthaflavone caused approximately a 100% increase, with a shift from cytochrome P-450 to cytochrome P-488. In contrast, β-naphthaflavone caused a greater increase (175%) in cytochrome C reductase (NADPH) than phenobarbital (41%). This latter change contrasts with that of the rat, in which typical P-448 inducers do not cause increases in the reductase activity. The increases were accompanied by the expected increases in enzyme activity: aldrin epoxidase was increased by phenobarbital, 7-ethoxyresorufin deethylase was increased by 6-naphthaflavone, and 7-ethoxycoumarin was increased by both. Duignan et al. (1987) used a step-wise regimen (to avoid excessive sedation) to induce beagle dogs with phenobarbital (Na+ salt): 10 mg/kg (po) for 2 days, followed by 30 mg/kg for 4 days, then 30 mg/kg for a final 8 days. This regimen more than doubled the microsomal concentration of cytochrome P-450 as well as significantly increasing the activities toward 7-ethoxycoumarin, warfarin, and androstenedione. There were changes in region and site specificity that indicate that induction in the dog, as in the rat, is accompanied by shifts in isozymic character of cytochrome P-450.

Aldrich and Niems (1979) examined the effects of phenobarbital and 6-naphthaflavone on the metabolism of caffeine in vivo in the dog. Both phenobarbital (10 mg/kg/day po for 7 days) and β-naphthaflavone (20 mg/kg/day ip for 3 days) decreased the half-life of caffeine, but only β-naphthaflavone caused a qualitative shift in the spectrum of urinary metabolites.

Gascon-Barre et al. (1986) studied the effects of phenobarbital induction of the MMFO on vitamin D metabolism in mongrel dogs. Dogs were given approximately 80 mg/kg/day for 30 days and induction was monitored by following changes in vivo [^{14}C]aminopyrine metabolism

[14]C-C0$_2$ production. The paper does not mention complications due to the sedative effects of phenobarbital. As might have been expected in an outbred population, some dogs were more inducible than others. In fact, in two dogs, no induction occurred at all. Not surprisingly, induction resulted in increased hepatic catabolism of vitamin D$_3$. The important points to be stressed here are that variable responses of dogs to inducing agents can be a potential problem and that in vivo clearance of aminopyrine could provide a noninvasive probe for screening of "good" versus "poor" responders to inducing agents of the phenobarbital class.

The inducing effects of drugs and chemicals other than of phenobarbital and β-naphthaflavone (typical experimental tools) have also been studied in the dog. Lan et al. (1983) compared and contrasted the inducing effect of hexahydroindazole (10–250 mg/kg/day for a month) in three species. Increases in relative liver weights occurred in all three species but increases in microsomal protein only in rats and monkeys (and not the dog). Increases in cytochrome P-450 and aminopyrine metabolism occurred in all three species, but the largest increases (compared to concurrent controls) were observed in dogs.

Abramson et al. (1986) and Abramson and Lutz (1986a,b) have extensively studied the relationship between enzyme induction, in vivo antipyrine metabolism, and increases in alpha-1 acid glycoprotein. While the relationship between the latter two parameters is not a simple one, they can be used to monitor the extent of induction by phenobarbital-type agents in the dog. Using these techniques, they demonstrated that phenytoin (Abramson and Lutz, 1986a) and rifampicin (Abramson and Lutz, 1986b) are effective inducing agents in the dog, while medroxyprogesterone was not (Abramson et al., 1986). The latter finding is of interest because medroxyprogesterone has been reported to be an inducing agent in rats. This underscores again the point that there are species-related differences in responses to inducing agents.

Epoxide hydrolase is an important enzyme in the metabolism of reactive arene oxides, but up until 1980, little work had been done to characterize this enzyme in the dog. In 1981, Pacifici et al. reported the activity of hepatic microsomal epoxide hydrolase (with styrene oxide) in the dog to be 9.7 ± 2.0 nmol/min/mg protein, which was intermediate between that of the mouse 1.9 nmol/min/mg and the baboon (31.3 nmol/min/mg). Gregus et al. (1983) confirmed that the dog has relatively high epoxide hydrolase activity (approx. 15 nmol/min/mg protein) compared to many other commonly used laboratory species, including the rat. Little other work has been completed to distinguish, either on structural or substrate specificity basis, epoxide hydrolase of the dog from that of other species. Given its high activity, however, it can be expected to play an important role in xenobiotic metabolism in the dog.

As in other species, conjugative metabolism (other than mercapturic acid formation) has been studied in the dog longer than oxidative metabolism. As reviewed by Hirom et al. (1977), the majority of the conjugative reactions were described in Germany during the latter part of the nineteenth century. The dog, like most mammals, excrete phenols as sulfates and glucuronides. In the dog, the ratio of sulfate to glucuronide (at an aromatic hydroxyl group) varies with substrate. For example, with phenolphthalein, the dog will excrete 18% as the glucuronide and 82% as the sulfate (as reviewed by Hirom et al., 1977), whereas with acetaminophen, 75% will be excreted as the glucuronide and 10%–20% as the sulfate (Hjelle and Grauer, 1986). With aryl acetates, the dog has a high tendency to form conjugates with amino acids. For example, with benzoic acid, the dog will excrete 82% as the glycine conjugate (hippuric acid) and only 18% as the glucuronide.

Relatively little work has focused on the biochemical and molecular characterization of the enzymes involved in conjugation in the dog. Gregus et al. (1983) examined the in vitro activity of PAPS-sulfotransferase of the dog against four substrates. Activity was noted with all four: the highest with 2-naphthol and the lowest with taurolithocholate. When compared to other species, the sulfotransferase activity of the dog tended to be lower than most.

Gregus et al. 1983 examined the activity of UDP-glucuronosyl transferase against a wide variety of substrates. The dog had somewhat higher activities than the rat with 1-naphthol, p-nitrophenol,

estrone, morphine, and digitoxigenin-monodigitoxoside, whereas the opposite was true with diethylstilbestrol and bilirubin. Hence, in terms of relative activity, there is little to distinguish the UDP-glucuronosyl transferases of the dog from those of other species. There is evidence, however, of species differences in stereoselectivity. Wilson and Thompson (1984) examined the stereoselectivity of dog hepatic microsomal UDP-glucuronosyl transferase by examining differences in activity toward (R)- and (S)-propranolol. When racemic mixtures were studied, the (S) enantiomer was the preferred substrate, with 3–4 times more (S)-propranolol glucuronide produced than that of the (R) enantiomer. Additional experiments with separate enantiomers demonstrated that the K_m and V_{max} of the dog microsomal UDP-glucuronosyl transferase are much higher with the (S) than the (R) enantiomer. In human subjects, plasma levels of (R)-propranolol have been shown to be higher than those of (S)-propranolol (Silbert et al. 1982), whereas the opposite has been shown in dogs (Walle and Walle, 1979), and this difference may be due to the differences in stereospecificity of glucuronidation in the dog versus human (Von Bahr et al., 1982). Stereospecificity of UDP-glucuronosyl transferase with substrates other than propranolol has been reported for other species (Sisenwine et al., 1982). Thus, stereospecificity of UDP-glucuronosyl transferase is not unique to the dog or propranolol.

Schmoldt et al. (1987) examined the in vitro glucuronidation rates of dog liver microsomes toward various cardiac glycosides. In contrast to most other species examined, dog UDP-glucuronosyl transferase is capable of conjugating digitoxin; otherwise, glucuronidation rates between rats and dogs were similar for all other cardiac glycosides examined. In fact, neither species has detectable activity toward digoxin. As both dog and humans rapidly eliminate (in vivo) administered doses of digoxin, these results suggest that there may be greater similarity between canine and human UDP-glucuronosyl transferase than between the human and rat enzyme with regard to substrate specificity. UDP-glucuronosyl transferase exits as a family of different isozymes in rats (Knapp et al., 1988). By examining a variety of model substrate and various inhibitors, Schmoldt et al. (1987) concluded that while there may be more than one canine isozyme, a single isozyme in the dog was responsible for glucuronidation of all cardiac glycosidase.

The dog has long been recognized to have less active N-acetyl transferase activity than other species (Williams, 1971). This was more recently confirmed by Gregus et al. (1983), who demonstrated that the dog had almost imperceptible activity with five different substrates. This is a qualitative species difference that could result in toxicologically important species-related differences in metabolism. For example, arylamines require acetylation to be activated to mutagens and carcinogens. In an interesting paper, Neis et al. (1985) compared the cytochrome P-450 content, N-acetyltransferase activity, and mutagenic activation activity of canine and human isolated hepatocytes. The mutagenic activation of five different arylamines was examined, and it was found that human hepatocytes had much greater mutagenic "activation" activity; this difference was attributed to the N-acetyltransferase present in human cells. Paroxon, an inhibitor of acetyl transferase, decreased mutagenic activation by human hepatocytes. They noted incidentally that dog hepatocytes were approximately the same size as human hepatocytes, but those of the dog had higher concentrations of cytochrome P-450: 210 ± 10 versus 94 ± 2 pmol/10E6 cells. N-Acetylation is thus involved in the activation of arylamines in humans, and therefore the dog may not be the appropriate model in which to study the toxicity (relative to humans) of arylamines.

Arylamines are not only substrates of the MMFO, but they can also be substrates for the flavin-based mixed function oxidase (FMFO). The FMFO of the dog has not been isolated and well characterized like that of the pig or the rat, but differential arylamine metabolism and the use of specific inhibitors has provided a probe for studying the involvement of this enzyme in dog xenobiotic metabolism. 2,(2,4-Dichloro-6phenyl)phenoxy-ethylamine (DPEA) is a specific cytochrome P-450 inhibitor and methimazole is a competitive inhibitor of FMFO. Using these, Hammons et al. (1985) have reported that the FMFO metabolized 2-acetylaminofluorene in pigs and humans, but not in dogs and rats. This group also studied the in vitro metabolism of 1-naphthylamine (1-NA) and 2-naphthylamine (2-NA) in rats, dogs, and humans. N-Hydroxylation is a major pathway in all

three species for 2-NA, but not for I-NA, and this reaction is exclusively mediated by the MMFO. Ring hydroxylation, however, also occurred in all three species and may be partially mediated by the FMFO as well as the MMFO because it was not completely inhibited by DPEA. For both chemicals, the dog has a higher rate of metabolism than the rat. Interestingly, there were greater individual human-to-human differences in the microsomal metabolism of these chemicals than the differences between rats and dogs.

Like all known and studied mammals, dogs have hepatic cytosolic glutathione S-transferase (GSH-T). Grover and Sims (1964) described this enzyme in dogs as early as 1964 and were the first to note the high activity the dog GSH-T has with 1,2-dichloro-4-nitrobenzene. Gregus et al. (1983) examined GSH-T activity against six different model 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene; otherwise, rats have much higher activity than dogs with all other substrates examined.

In 1986, Wiener confirmed in mongrel dogs the observation as to GSH-T activity toward 1,2-dichloro-4-nitrobenzene and further compared the activities of rat and dog cytosolic preparations against seen in other substrates and found that the activity in the dog ranged from 2.5% (4-nitropyridine N-oxide) to 95% (ethacrynic acid) of those of the rat. Overall, the highest activity in dogs (and rats, for that matter) was obtained with 1-chloro-2,4-dinitrobenzene. Weiner further characterized GSH-T in various cytosolic protein fractions. He found that dog GSH-T exists as four different isozymes composed of three different classes of subunits. In general, this type of isozymic pattern for this enzyme has been seen in other species. The dog enzymes are also not very different with regard to sensitivity to inhibitory ligands, such as bromocresol green or 8-anilino-1-naphthalene (Wiener, 1986). Therefore, except for the expected molecular and quantitative activity differences, GSH-T in the dog is not startlingly different from that of other species. Weiner did observe that dog GSH-T has some activity in the denitrification of organic nitrates, such as isosorbide-2,5-dinitrate, and that this activity was responsible for the rapid in vivo conversion of 2,3-di-O-nitro-adenosine-5′-(N-ethyl-carboxamide) to the mono-nitro chemical in the dog (Wiener et al., 1983). Whether this is a pathway unique to the dog remains to be established.

In general, little in-depth work on extrahepatic metabolism in the dog has been reported, the exception being work done at the Lovelace Inhalation Toxicology Research Institute on the respiratory tract. Hadley and Dahl (1982) studied the cytochrome P-450-dependent monooxygenase activity in the nasal membrane of several species. The dog tended to have the lowest amount of cytochrome P-450 and the lowest MMFO activities.

Bond et al. (1988) characterized the cellular and regional distribution of xenobiotic metabolizing enzymes in the respiratory airways of beagles and found detectable cytochrome P-450-dependent activity throughout the airway, from the alar fold to the peripheral lung, but there were regional and substrate differences in specific activities. For example, the ethmoid rubinate had high activities for both benzo(a)pyrene and ethoxycoumarin metabolism, while higher nasal regions (alar fold, naso-turbinate and maxilloturbinate) had much higher activity with ethoxycoumarin. In general, epoxide hydrolase and GSH-T activities were present along the entire airway but tended to be higher in the nasal region and pulmonary airways compared to the major airways. UDP-glucuronosyl transferase tended to be evenly distributed. These "detoxification" enzymes were present in much greater activity than the MMFO-cytochrome P-450-dependent "activation" system. This work provides an excellent basis for using the dog in the study of site-specific chemical carcinogenesis of the respiratory tract.

For example, Petridou-Fischer and colleagues (1987) studied the in vivo disposition of nasally instilled dihydrosafrole in the dog and recovered metabolites from the nasopharyngeal mucus, thus confirming the potential importance of the MMFO activities in the upper respiratory tract in the metabolic activation of potential carcinogens.

Other aspects of extrahepatic metabolism in the dog have been partially investigated as part of studies of various species-related differences in target organ toxicity. Garst et al. (1985) examined the pulmonary metabolism of perilla ketone by a variety of species and concluded that the

insensitivity of the dog lung to perilla ketone toxicity resulted from the relatively low amount of cytochrome P-450 with low MMFO activity toward perilla ketone.

Poupko et al. (1983) reported that the dog bladder microsomes had very low activity with both 1- and 2-naphthylamine.

Menard and colleagues (1979) examined both testicular and adrenal cytochrome P-450 in the dog, guinea pig, and rat. The dog and rat had comparable concentrations of adrenal cytochrome P-450 (1.1–1.2 nmol/mg microsome protein), whereas the guinea pig had roughly twice this concentration. In contrast, the dog had the highest concentration of testicular cytochrome P-450 (0.170 nmol/mg microsomal protein) and the rat the lowest (0.067 nmol/mg microsomal protein). Administration of spironolactone, a 7-{alpha}-thiosteroid, led to the destruction (40%–60%) of the cytochrome P-450s of both tissues in the dog. In contrast, spironolactone had no effect on adrenal cytochrome P-450 in the rat but caused large decreases (88%) in testicular cytochrome P-450.

Pacifici et al. (1981) reported on species and tissue difference in epoxide hydrolase and GSH-T and found the epoxide hydrolase activity of the dog kidney to be comparable to that of the guinea pig and rabbit, but less than that of the hamster. GSH-T activity of the kidney of the dog (with styrene oxide, the same substrate used to determine epoxide hydrolase activity) tended to be much lower than those of the guinea pig and rat but higher than those of the human and monkey. There are species differences in extrahepatic cytochrome P-450 of which the investigator should be aware, but it does not appear that the dog offers advantages over any other species in the study of specific routes of extrahepatic metabolism.

Very little work has been reported on developmental or age-related changes in drug metabolism in the dog. Tavoloni (1985) examined the age-related development of the cytochrome P-450 MMFO and UDP-glucuronosyl transferase in the beagle dog. Activities were lowest at birth. They increased thereafter, tending to plateau after 6 weeks of age. Increases in the MMFO, but not UDP-glucuronosyl transferase, were induced by phenobarbital.

Baarnhielm et al. (1986) compared and contrasted the in vivo and in vitro metabolism of felodipine in the rat, dog, and human. Felodipine is a calcium channel blocker that is very lipophilic, well absorbed from the gastrointestinal tract, highly protein bound, and extensively metabolized. Comparative in vitro rates of microsomal metabolism of felodipine were rat > dog > human. There was an excellent correlation between the V{max}S and the concentrations of cytochrome P-450. The same rank order was observed for in vivo plasma clearance. It would appear that with chemicals that are well absorbed and rapidly metabolized, interspecies extrapolations are possible and fairly predictive. In their studies on the disposition of etodolac, Cayen et al. (1981) observed that felodipine also was well absorbed and partially metabolized (different metabolites in dog and rat) and undergoes extensive intrahepatic circulation. There were differences in the serum half-life (human > dog > rat) and clearance (dog > human > rat), but there was greater similarity between the dog and human. In general, the dog appears to be a better pharmacokinetic model (more predictive of behavior in human subjects) for humans than the rat or other rodents for chemicals that are well absorbed and have a high hepatic clearance.

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Primates

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CONTENTS

TOXICOLOGY

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The phylogenetic and physiological similarity between humans and nonhuman primates has resulted in an increased demand for certain species in safety and efficacy assessments of new drugs and biologics. Only a few of almost 200 primate species are utilized in toxicology studies. The most commonly used species are old-world monkeys (cynomolgus monkey, rhesus monkey, and baboon) and new-world monkeys (squirrel monkey and marmosets).

Because of the genotypic and phenotypic resemblance to humans, nonhuman primates have been used in the study of induced or naturally occurring human diseases such as acquired immuno-deficiency syndrome (AIDS), hepatitis, diabetes mellitus, and atherosclerosis. Our understanding of the human brain, vision, aging, reproductive function, and behavior has been enhanced by studies in primates. Safety (and sometimes efficacy) evaluations of drugs, vaccines, and biotechnology

products are conducted in nonhuman primates prior to approval for general use by the public. A failure to investigate the potential teratogenic effects of thalidomide in a primate model prior to exposing pregnant women resulted in tragic consequences in the 1950s and early 1960s (Somers, 1963). Unfortunately, the teratogenicity of thalidomide could not be demonstrated in rodents prior to human exposure, but was subsequently shown to cause fetal abnormalities in primates (Hendrickx et al., 1966; Hendrickx, 1973). The development of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) has been a significant advancement for the treatment of hypercholesterolemia (and related hyperlipidemias). The withdrawal of a popular drug in this class, cerivastatin, highlighted the importance of preclinical toxicology in rodent and nonrodent species, as the development of life-threatening rhabdomyolysis in humans ultimately was associated with differences in animal model (and human) pharmacokinetics (PK) among drugs in the class as well as multiple drug therapy and renal insufficiency in the target population (Evans and Rees, 2002).

In spite of the obvious predictive value of primates for human responses, they must be utilized conservatively, and models in lower species should be used when appropriate. The majority of nonhuman primates used in biomedical research originate from international and domestic source, purpose-bred breeding colonies. To a much lesser extent, primates are ethically obtained from large feral populations; the animals are imported or domestically bred. Special handling procedures resulting from the developing body of knowledge about the zoonotic potential of known (and emerging) infectious diseases to humans create unique challenges and increase the cost associated with the use of nonhuman primates. Nevertheless, the humane use of nonhuman primates as a physiological, pharmacological, and toxicological research model is critical for safety assessment of new drugs and biotechnology products, increasing the need for prudent use of primate inventories.

The intent of this section is to define the utilization of nonhuman primates in toxicology studies and to describe the basic husbandry and technical procedures used in a primate facility. It is not intended to be an exhaustive review of the voluminous primatology literature, but rather an overview of selected topics and considerations. Additional comprehensive references on both the biology and roles of nonhuman primates are available and provide comprehensive information with regard to this subject.

Husbandry

Husbandry includes all aspects of housing and adequate care. Primates encompass a variety of species ranging from lower forms such as the tree shrews to the great apes. Much in the literature has generalized the nonhuman primate as one animal when in reality the primate can be as small as several grams (e.g., shrews) to over 100 kg (e.g., great apes). The primary genus utilized in toxicological studies has been the macaque, with lesser emphasis on the new-world monkeys. Although the chimpanzee has made significant contributions to biomedical research (and continues to be used in small numbers), proposed legislation will make this species increasingly difficult to utilize as a research model. The primary focus of this section will be on the macaque spp. with specific reference when necessary to new-world primates.

Federal regulations enacted through the Animal Welfare Act, Parts I, II, and III, further define the need to provide optimal caging, adequate veterinary care, protocol development through scientific and animal care committee review, and programs geared toward a variety of diverse procedures in assuring the physical and psychological well-being of the primate. In addition to welfare regulations concerning housing and care, the nonhuman primate requires special enrichment programs to assure that the animals are psychologically enriched and display species-specific behaviors. Social housing of nonhuman primates, ranging from periodic commingling, pair housing, and/or group housing, has become standard in toxicology research. Social housing of primates must be the default for all research programs; however, the study design (to include objectives, route of dosing, and known test article effects) may require the attending veterinarian to provide an

exemption to social housing. Special caging and compatibility assessments are needed for successful social interactions with the animals. By matching the commingled animals by dose group and scheduling the interactions to avoid interference with study procedures, successful social housing can be accomplished with almost any study. Nonhuman primates are highly intelligent, and successful environmental enrichment requires novel presentation of toys, puzzles, and other devices rotated frequently enough to maintain the interest of the animals. Decreased stress and more normal physiological responses are the rewards of a carefully designed socialization and environmental enrichment program.

Institutional Policy and Regulatory Issues

Institutional animal facilities and programs should be operated in accordance with the Animal Welfare Act and as described in the *Guide for the Care and Use of Laboratory Animals* (The Guide, 2011) and other applicable federal, state, and local laws, regulations, and policies. Nothing in the regulations intends to restrict the investigator's freedom to conduct animal experiments in accordance with scientific or humane principles. Any program of husbandry must be developed considering the proper care and humane treatment of the animals used in research or testing. Aspects of conservation, alternatives to animal use, elimination of experimental duplication, and strong scientific principles must be paramount in experimental design.

The U.S. Department of Agriculture (USDA) Animal Welfare Regulations (Federal Register, 1989) and the Public Health Service through the Office for Protection from Research Risks of the National Institutes of Health (NIH/OPRR) mandate the appointing of an Institutional Animal Care and Use Committee (IACUC) to review the humane principles of research protocols and the total Animal Care and Use Program of each research institution. Included in this review are semiannual facility and program assessments that cover proposed and ongoing animal use activities, veterinary care program, training, standard operating procedures (SOPs), pain and distress alleviation, and methods of euthanasia. As a part of the assessment, the IACUC is required to submit a semiannual written report to the institutional official on the status of the Institutional Animal Care and Use Program.

Facilities

Physical Plant

A key to a successful toxicological research program is the design of the animal facilities. A good design assures efficiency in animal care and personnel movements and provides managers with the facilities necessary for an economic and sound animal care program. A facility design should afford the flexibility to conduct studies of various types and projects that vary in animal numbers.

Briefly, the following functional areas are essential to assure that diverse projects, different primate species, dosing and collection requirements, and husbandry and sanitation needs can be met (The Guide, 2011):

1. Separation of primate housing from administrative or human occupancy areas. Areas with animal procedure rooms (as distinguished from laboratories) can be adjacent to basic animal housing areas.
2. Special areas such as quarantine or receiving require isolation from primary study rooms or laboratories to assure project contiguity and ensure animals meet the required health specifications for the study.
3. Separate areas for surgery, necropsy, postsurgical care, clinical pathology, radiography, diet preparation, or animal treatment.
4. Office areas for administration and facility support areas, separate from animal areas.

5. Support areas for personnel, e.g., breakroom, lockers, showers, and toilets.
6. Feed and supply storage and receiving areas.
7. Caging storage, general equipment and supply storage, washing facilities, and incinerator or waste disposal.
8. Central supply and receiving.

The size and complexity of these areas depend on the size of the facility. A facility less than 1000 ft² has significantly different requirements from a facility housing numerous primates larger than this size. Efficiency and economy in utilization of research workers' time must be carefully balanced with the need to separate animal and human facilities. Careful consideration of personnel and animal traffic patterns must be utilized in the design of animal facilities. Separate housing areas will be required for research programs that utilize various primate species. Security has become a key issue in animal facility design. Barriers, entry locks, separate corridors, and/or separate floors all can enhance security.

Building Materials

Economy, maintenance, and sanitation must be considered in the selection of materials for the research facility. Key features of an animal room are durability, esthetics, ergonomics, and ease of sanitation procedures while providing a humane and comfortable housing area free of pathogens and vermin.

Construction Criteria

The design of primate housing areas is unique. Basic aspects to consider include the following:

1. Corridor widths should be of adequate size to permit easy movement of caging to wash area and bumper guards to prevent wall or corner damage.
2. Animal room doors with viewing windows, swinging toward the corridor only when an anteroom is present. Preferable construction is solid or metal doors to eliminate vermin-hiding places. The doors accessing animal areas should have self-closing devices.
3. Floors should be of durable, sanitary, nonslip construction, e.g., epoxy-sealed concrete or suitable substrate.
4. Walls of sealed impervious paints or coatings using coved and sealed junctions at floor and ceiling are required.
5. Ceilings should be of nonpermeable construction or sealed with washable coatings. Light fixtures of waterproof-sealed construction are required, with adequate light dispersion to allow for animal observations at all cage levels.

Ventilation, Temperature, and Humidity Control

Environmental control is an essential component of facility design in a toxicology laboratory. Nonhuman primates have specific requirements for ventilation, temperature, and humidity. Therefore, environmental consideration must always be given to the specific species being housed. For example, new-world primates are specifically affected by low humidity (less than 50%), which may contribute to upper respiratory afflictions (The Guide, 2011). Ventilation rates of 10–15 air changes per hour are essential to reduce environmental aerosol contamination as well as to provide odor control and minimize air ammonia concentrations. Recirculation of air is not advised unless it has been treated to remove particulate or toxic gaseous contaminants (Besch, 1980).

Temperature control systems are required to assure appropriate ranges of temperature from 64°F to 84°F (18°C–29°C) (The Guide, 2011). The two most important considerations in the physical

environment of primates are humidity and temperature as they closely correlate to metabolism and behavior. Humidity is recommended at between 30% and 70% for old-world species. The toxicological laboratory must be more sensitive to temperature or humidity variations because such finite measurements as cellular parameters and hormonal indices can be affected by environmental variances (Gortan and Besch, 1974).

Power and Lighting

Steady and uninterrupted power with backup emergency generators is essential to assure critical environmental controls are maintained. Lighting of sufficient intensity is necessary to provide good husbandry and sanitation practices, observation of animals, security, and safe working conditions. A biphasic or a variable intensity lighting system may be necessary in certain facilities to provide adequate observations, yet provide soft diffused lighting for the majority of the day. Illumination of 75–125 foot-candles (Bellhorn, 1980) has been recommended for observation periods. Diurnal time-controlled systems are essential in a research animal facility to provide an environment that promotes regular circadian rhythms.

Lights should be sealed and preferably of fluorescent type for energy efficiency. Some facilities have converted to the daylight-colored ultraviolet (UV) light fixture to simulate natural lighting; either is acceptable and appropriate for the subject species.

Drainage

Adequate waste control is essential to assure that contamination, odor, and waste stoppage of drains do not occur. Research colonies are at increased risk of disease transmission from sewage overflow. All waste fixtures should be of adequate size to permit full flow of waste biscuits, feces, hair, and similar materials. Drain sizes from 4 to 6 in. are recommended (The Guide, 2011) with appropriate floor sloping to facilitate the removal of water and waste materials. Cages should be provided with flush pans and troughs to direct and keep waste off of the common pathways. Facilities using dry paper or bedding systems should have adequate disposal systems for these materials. Lockable drain covers or appropriate-sized grids may be necessary to prevent improper materials from being deposited into (or obstructing) the drain system.

Storage Areas

A variety of storage areas are necessary to allow effective husbandry procedures to be performed. These include clean food and bedding storage, temperature-controlled food storage to prevent vitamin and nutrient deterioration, refuse storage areas for dry bedding or paper, and equipment storage for clean or dirty caging, disinfectants, and personnel items (e.g., masks, gloves, etc.). Consideration should be given to the myriad of equipment (e.g., precision scales, electrocardiogram [ECG] machines, blood pressure monitors, and infusion pumps) that are required to support the conduct of study procedures. Most of this equipment must be stored in a dedicated area that remains clean and dry or be configured to be resistant to laboratory and animal room conditions.

External Environmental Influences

The animal facility should be free of unusual external influences since glandular and immunologic functions can be affected by sudden or unusual noise. Cage washing areas should be separate and apart from animal rooms. Soundproofing materials should also be utilized to prevent unwelcome noise in the animal facility (Fletcher, 1976; Peterson, 1980). Large primates can generate significant noise pollution by shaking their cages. Cage anchors may be necessary to minimize cage

movement and damage to facilities. These animals should be housed an appropriate distance from more sensitive studies and animal species.

Special consideration is required for primate behavioral and/or hormonal evaluations. External influences, such as turning on a light or sudden personnel intrusion during nighttime periods, can adversely impact these studies. Fire alarm tests should be done with muffled alarms in the animal area to prevent severe animal distress due to the noise levels.

Sanitation Facilities

Critical to the husbandry of any facility are areas for washing and sterilizing animal cages, racks, water bottles, and similar accessories. The USDA/Animal Welfare Regulations mandates the sanitation/washing of cages at a minimum of every 2 weeks. Temperatures not lower than 180°F are recommended with use of appropriate disinfectants (The Guide, 2011). Key factors to consider in the proper sanitation facilities include

1. Location related to traffic, animal rooms, elevators, and waste disposal
2. Soundproofing
3. Utilities such as hot and cold water, steam, drainage, and power
4. Proximity to storage and appropriate storage area to meet clean and dirty cage demands
5. Ventilation and employee safety
6. Access and corridor width

The size of the facility dictates whether manual washing using brushes and portable units is satisfactory or whether large automated washers are necessary.

Animal Rooms

Animal rooms for toxicological experiments should be variably sized to house either large or small numbers of animals and to provide project segregation during (at a minimum) the dosing phase. Usually this is best accomplished by having a variety of sizes of animal rooms. Laminar flow hoods and bubble-isolette room systems (such as the BioBubble®, BioBubble, Inc., Fort Collins, Colorado) have been designed to permit multiple studies in fairly large rooms.

In general, animal rooms should have an anteroom provided with a sink and area for outer garments storage (e.g., disposable coveralls, masks, gloves, and dedicated footwear). The animal room proper should be constructed with all of the specifications noted earlier to effectively permit a sanitary and humane environment (Figure 9.1). Recent concerns about psychological enrichment may dictate play areas, group housing areas, or special cage constraints to assure animal well-being and to provide the opportunity for exercise. Social needs of the nonhuman primate influence animal room design, research, and husbandry management techniques. All programs using primates should have primate environmental enrichment and some level of socialization incorporated into the facility design.

Support Areas

Personnel support areas, including appropriate break areas, locker rooms, and shower areas, are essential to any husbandry program. These areas should be carefully designed and managed for traffic flow to prevent risks to animals and personnel from contamination and disease.

Special Areas

Most facilities will have special requirements for aseptic surgery, involving pre- and postoperative care, surgeon scrub areas, and operating rooms; pathology and necropsy rooms; clinical

Figure 9.1   Nonhuman primate animal room. Floors and walls are made of nonporous materials that are readily sanitized. Watering systems are typically automatic.

pathology, radiology support, and treatment and procedure areas including facilities where aseptic procedures can be conducted; and a variety of other requirements, depending on the species involved in research and the specific techniques and procedures. The size and complexity of the facility will dictate the size of each of the aforementioned areas, but all are necessary parts in the support of the overall facility, husbandry, and management procedures.

Biohazard Areas

The study of drugs for the treatment of AIDS, hepatitis, and other infectious diseases, when there is need to use animals exposed to the live agents, dictates the need for special facilities to work with hazardous agents whether chemical, biological, or physical. Each project will dictate the need for the level of containment. The Centers for Disease Control (CDC) has listed four levels for dealing with potential biological hazards (from biological safety level [BSL]-1 to BSL-4), each with specific criteria. Special hoods, filters, negative room pressures, procedures, and personnel practices are involved. The reader is referred to the CDC (2009) publication on *Biosafety in Microbiological and Biomedical Laboratories* for reference to this vital area.

Caging and Equipment

The well-being and health of the nonhuman primates is critical to the success of a research project. The caging or housing system must be designed to assure that the research objectives and species-specific needs can be met through minimizing experimental variables and assuring the "normality" of the animal via the maintenance of health and well-being. The Guide (2011) provides the following factors to consider in any housing system:

1. Provide adequate space to assure freedom of movement and normal postural adjustments with a resting place (perches).
2. Ensure a comfortable environment.
3. Utilize escape-proof caging.
4. Provide easy access to food and water.
5. Supply adequate ventilation.
6. Meet the biological and environmental needs of the species.
7. Keep animals in a dry and clean environment.

8. Reduce unnecessary physical restraint.
9. Protect animals from known chemical, biological, and physical hazards.
10. Provide options for environmental enrichment (e.g., grooming bars, perches, and commingling or social housing enclosures).

Individual Housing

Cages for nonhuman primates must meet the space recommendations noted in The Guide. Table 9.1 lists the current space requirements provided in this document.

Institutions are encouraged to provide alternatives to individual caging and incorporate age- and sex-related and behavioral characteristics into a plan to facilitate the alternatives (group housing, pairs or triplets). Infants and juveniles can be housed in group cages, for example, with very few complications associated with sex-related and behavioral characteristics. If adults are to be housed in groups, it is essential that only compatible animals be kept together (Lee, 2013). Newly grouped animals must be closely monitored to detect injuries due to fighting. Space in group cages should be enriched with structures such as resting perches at multiple heights, multiple feeders and water sources, visual barriers, and shelters. Vertical space is equally important as floor space in arboreal species such as the macaque. For chimpanzees and brachiating species (orangutans, gibbons, spider

Table 9.1   Individual Housing Chart Needs to Be Updated

| Animals | Weight (kg) | Type of Housing | Floor Area/Animal | | Height[a] | |
			ft²	m²	in.	cm
Nonhuman primates[b]						
Group 1	Up to 1.5	2.1 (0.20)	30 (76.2)	Group 1	Up to 1.5	2.1 (0.20)
Group 2	Up to 3	3.0 (0.28)	30 (76.2)	Group 2	Up to 3	3.0 (0.28)
Group 3	Up to 10	4.3 (0.4)	30 (76.2)	Group 3	Up to 10	4.3 (0.4)
Group 4	Up to 15	6.0 (0.56)	32 (81.3)	Group 4	Up to 15	6.0 (0.56)
Group 5	Up to 20	8.0 (0.74)	36 (91.4)	Group 5	Up to 20	8.0 (0.74)
Group 6	Up to 25	10 (0.93)	46 (116.8)	Group 6	Up to 25	10 (0.93)
Group 7	Up to 30	15 (1.40)	46 (116.8)	Group 7	Up to 30	15 (1.40)
Group 8	>30[e]	≥25 (≥2.32)	60 (152.4)	Group 8	>30[e]	≥25 (≥2.32)
Chimpanzees (Pan)						
Juveniles	Up to 10	15 (1.4)	60 (152.4)	Juveniles	Up to 10	15 (1.4)
Adults[f]	>10	≥25 (≥2.32)	84 (213.4)	Adults[f]	>10	≥25 (≥2.32)

Notes: Cage height should be sufficient for the animals to comfortably stand erect with their feet on the floor. Baboons, patas monkeys, and other longer-legged species may require more height than other monkeys, as might long-tailed animals and animals with prehensile tails. Overall cage volume and linear perch space should be considerations for many neotropical and arboreal species. For brachiating species, cage height should be such that an animal can, when fully extended, swing from the cage ceiling without having its feet touch the floor. Cage design should enhance brachiating movement.

For other apes and large brachiating species, cage height should be such that an animal can, when fully extended, swing from the cage ceiling without having its feet touch the floor. Cage design should enhance brachiating movement.

[a] To convert kilograms to pounds, multiply by 2.2.

[b] Singly housed primates may require more space than the amount allocated per animal when Group 1 marmosets, tamarins, and infants of various species group are housed.

[c] From cage floor to cage top.

[d] Callitrichidae, Cebidae, Cercopithecidae, and Papio.

[e] Larger animals may require more space to meet performance standards (see text).

[f] Apes weighing over 50 kg are more effectively housed in permanent housing of masonry, concrete, and wire-panel structure than in conventional caging.

[g] The interpretation of this table should take into consideration the performance indices described in the text beginning on page 55.
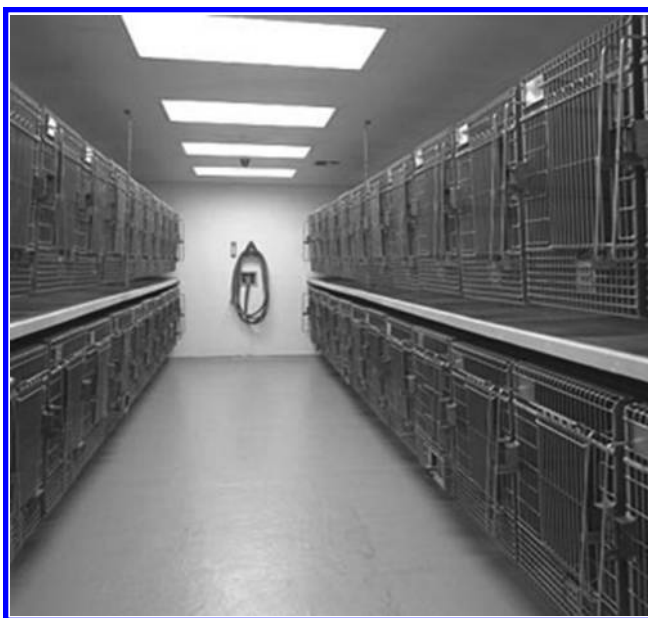
Figure 9.2   Rolling rack for housing multiple nonhuman primates. Commingling for animals in adjacent cages is provided; additionally, there are devices that can be attached to the front of the cage to allow vertical commingling.

monkeys, and woolly monkeys), the minimum cage height should be such that the animals can, when fully extended, swing from the cage ceiling without having their feet touch the floor.

Historically, cages have been constructed of a variety of materials. Caging must be constructed of sturdy durable materials and designed to reduce the possibility of contamination to adjacent cage units. All surfaces such as feeders and perches should be smooth and free from sharp edges or broken wires. A minimum of ledges, corners, or angles is recommended to prevent dirt or fecal retention. The cage squeeze device should be easily operable to avoid primate and personnel injury and for ease of use by the technical staff. The cage should facilitate animal observations and ergonomically correct and provide feeding access and appropriate watering devices. Cage designs have included wall-mounted floor, rolling rack (Figure 9.1), and permanent installations (Figure 9.2), all of which are satisfactory depending on species and project needs. The cage requirement for an adult chimpanzee is much different than for the new-world monkey, mostly a function of space and strength of design.

Social or Pair Housing

The benefits of social housing primates in toxicological studies are numerous. Psychological well-being programs should have written procedures for compatibility assessment, sexual maturity, gender separation, and cage security. Nonhuman primates are highly social, and interaction with other animals best satisfies this need and reduces stress. Pair housing when study procedures allow, or periodic commingling of compatible animals within the same dose groups, has effectively been used during toxicology studies. Special caging has been designed with sliding doors to intermittently connect cages to allow access between cages or grooming bars to allow tactile interaction if full-cage access cannot be allowed. Pair housing may also be used during periods of nonobservation or during nighttime hours on some studies. Animals can be housed socially in larger groups when on study using larger indoor pens. Consideration must be

Figure 9.3 Indoor pen housing system. This configuration meets the requirements for European nonhuman primate enclosures, which include a need for group housing and vertical height requirements.

given to animal access, study logistics, and observations of physiological functions of the animal (e.g., elimination, appetite, activity, etc.) (Figure 9.3).

Group caging has its own set of requirements that by necessity must consider group dynamics, animal access, behavioral needs, usable surface areas, volume versus square footage, escape areas, and management practices (Figure 9.4).

Equipment Ancillary to the Cage

Equipment necessary to move the cage, handle the animal, and maintain safety to the handler and animal is essential to the husbandry operation. This equipment can include cage lifts, transfer cages, transfer chutes for large social housing pens, and restraint apparatus (e.g., pole and collar, leather gloves, capture gun, nets).

Systems of Removing Waste

The two primary methods of waste handling are dry and wet methods. Dry methods involve the use of bedding or plastic or paper liners under the cage. This system permits aerosol-free waste removal and cleaning of animal rooms, yet has the disadvantage of excessive waste disposal and high labor requirements. This procedure for waste removal is sometimes employed, where there are heightened biosafety considerations (e.g., biological radioactive waste). The alternative system is the wet procedure using water under high pressure to move the waste down the trough to the floor drains. The advantage of this system is fast cleanup, but care must be taken to prevent aerosolization and potential contamination of adjacent animals and personnel. A modification of this method using a low-pressure manual or automatic flush system may be another approach. The use of wall-mounted proportionators allowing dual disinfectant/rinse flushing reduces contamination possibilities.
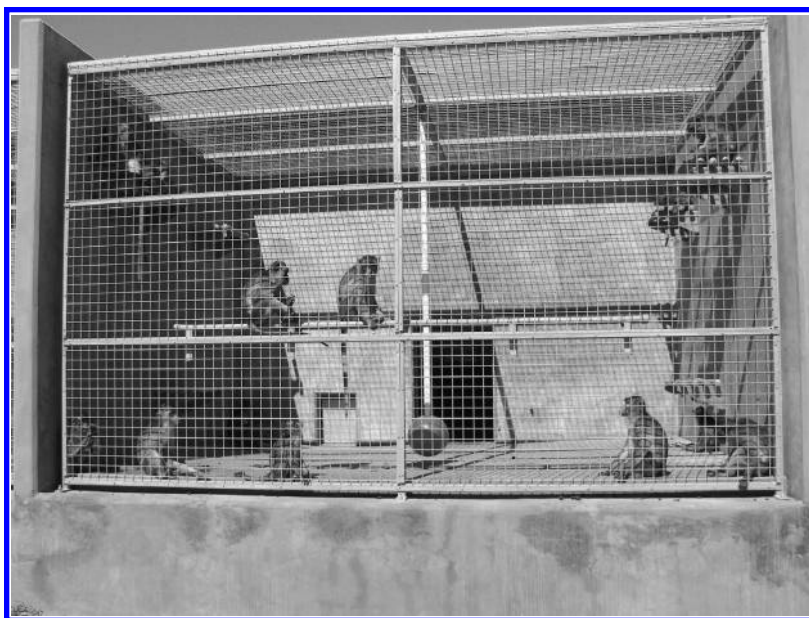
Figure 9.4 Outdoor animal enclosure system. These caging systems provide both an external environment and an internal area where environmental control is possible. The external caging area is protected from vermin and insects using various systems to bar entry.

Cages and Housing for the Great Apes

The most important considerations for housing the ape, specifically the chimpanzee, are strength, environmental and behavioral enrichment, space for adequate movement and exercise, and design simplicity. Group housing of chimps should be the default for captive chimpanzees. New rules for individual housing may require at least two times the current space recommendations for adult chimpanzees of 25 ft^2. Verifying this recommendation is needed. This would certainly have a direct impact on housing requirements and protocol design.

Environmental Enrichment and Special Concerns

Congress has coined the term "psychological enrichment" as a housing requirement for nonhuman primates (NIH, 2005). Essentially this has been meant to include environmental enrichment, social housing, behavioral well-being, and similar terms. Any primate research facility must take measures to assure that programs are in place utilizing and developing techniques to enrich the environment of primates regardless of assignment to a study. A written program for environmental enrichment is required by the USDA. Environmental enrichment includes the following:

1. *Social:* When possible and beneficial to the animal, all social species should be socially housed with emphasis on the appropriate type, size, and health status of pair or group. Social enrichment can be direct (e.g., pair or group housing) or indirect (e.g., objects that allow visual, auditory, or olfactory of conspecifics, also interaction with humans).
2. *Occupational:* Is also called cognitive enrichment, is task related, and requires the animal to use its mind to solve a problem; provides the animal with some control over its environment; can also involve/encourage exercise.
3. *Structural:* The actual physical design of the environment that the animal is housed in and the equipment or structural objects that can be added to it or changed within it to alter size or complexity.

4. *Sensory:* Stimulates one of the five senses comprised of visual, tactile, auditory, olfactory, or taste.
5. *Nutritional:* Provided through novel or preferred food items and various methods of food presentation/delivery. A variety of toys, puzzles, mirrors, etc., can be purchased or constructed for use in environmental enrichment and can be utilized in combination with food items. Care must be taken to assure that the equipment can be sanitized and to assure no parts of the equipment are hazardous. Use of occasional treats with human interaction or forage boards or as part of a puzzle feeder may be used. Such treats should not be of a quantity to adversely affect intake of a balanced diet, and certain research studies may limit the type or nature of the treats.

Nutrition and Water

Fortunately, there are adequate diets available commercially for nonhuman primates most commonly used in research. Most importantly, concern must be shown with the feeding techniques, feeding frequency, and feeding receptacles (numbers and types) used with primates. Careful observation of feeding behavior and appetite is essential. An empty feed cup does not signify a primate is eating because most of the biscuits may be on the floor nor does a half-empty feed cup indicate an animal is not eating adequately as each animal's metabolic rate is different. Regular monitoring of body weights and body condition scores are the best measures for determining the adequacy of the diet.

Physical Form and Presentation

The majority of commercial primate feed is produced through an extrusion process resulting, after baking, in a very hard product. A young primate may find the product to be unpalatable unless softened by soaking. It is important to provide the liquid used for moistening to the primate, as the vitamin C sprayed on the biscuit will be rinsed off if performed prior to offering the food.

Some toxicology studies require oral dosing using a specific vehicle. Careful attention to amount of vehicle, particularly on twice or three times a day dosing regimens, is required to avoid "filling" the primate up with resultant reductions in food intake and potential nutritional deficiency. Institutional standards are developed for nasogastric or oral dosing by species.

Careful attention should be given to dates of manufacture and rotation of feed stuffs to prevent deterioration of vitamin C. There are now diets containing stabilized forms of vitamin C, which allows the shelf life of commercial primate diets to be extended up to 180 days. It is important to confirm with the feed manufacturer the guaranteed shelf life and implement a rotation schedule appropriate to the manufacturer's recommendations. The food must remain palatable to the primate. Additionally, feed presented on the bottom of the cage without a feed cup may be contaminated by urine or feces, causing disease or iatrogenic anorexia.

Available Diets and Analysis

Commercial diets are usually adequate and can be followed with pre- or poststudy analysis. Certified diets are also available and are required by good laboratory practice (GLP) regulations. Analysis of noncertified commercial and noncommercial diets should be made to avoid potential contamination. It is incumbent upon the research facility to perform audits on food vendors to assure quality control programs are in place to prevent the contamination of diets. Special diets for special studies such as "low-sodium" or "high-fat" diets may be available commercially or may require custom preparation.

Food Restriction

It is common to fast the primate overnight prior to collection of clinical chemistry samples that may be affected by feeding or for preparation for anesthesia and surgery. The animals should not

be fasted for excessive periods in order to prevent hypoglycemia. This is especially problematic in young (less than 3 years of age) and pregnant macaques. Some level of food restriction may be necessary to facilitate dose level when bioavailability is affected by the presence of food in the stomach or proximal intestine. Longer-term food restriction during studies requires careful analysis to assure that animal well-being is not compromised (when balanced against potential scientific gains). Such modification to routine feeding practices must be justified to the facility's IACUC.

Water

Clean, fresh water should be available at all times and should meet or exceed the appropriate regulatory standards for drinking water. Modern husbandry systems provide water through filtered automatic water systems. Clean water bottles are also an acceptable method to provide water but require more monitoring and maintenance to assure sanitation and proper function of the bottle. Water can be a source of potential contaminants that can interfere with a variety of study results. Careful analysis for chemical and microbiological contaminants should be conducted routinely. Chemical testing includes analysis for contaminants (i.e., pesticides, heavy metals, and specific organic compounds). Special testing can be conducted at point of sampling if required for certain test article or studies.

Prevention of Disease and Injury

Procurement, Quarantine, and Conditioning

The assurance that the animal model used is of high quality and healthy is essential to any research program. This is particularly critical for nonhuman primates, which for the most part are obtained from breeding facilities internationally (e.g., China, Cambodia, Indonesia, Philippines, and Mauritius). Relatively few primates are bred in the United States for preclinical research. In order to prevent introduction of contagious conditions or zoonotic hazards into the animal colony, new animals should be quarantined in a set period of time. The CDC requires 31-day quarantine for imported primates, while 7–14-day quarantine is more appropriate for animals introduced from domestic sources. The primary reason for the quarantine length is to detect potentially latent diseases and prevent the introduction of these agents into a facility's existing primate colony.

1. *Procurement sources*: Most nonhuman primates used for research today are internationally purpose-bred animals. While some primates are feral caught, this represents a small proportion of the animals used in preclinical research. Nonhuman primates currently being utilized in preclinical research are purpose bred in a controlled environment, FI and F2 generations, and represent a superior health status. Cesarean-delivered or specific pathogen-free monkeys may be required on occasion, based on the study objectives and the known properties of the test article.
2. *Quarantine and conditioning procedures*: Because most of the monkeys used for research are transported from breeding centers and quarantine facilities internationally to the U.S. laboratories, quarantine and conditioning procedures are vital to assuring the health of the animals and to protect the laboratory colony from infectious diseases. A quarantine and conditioning program that pertains to the macaque will be described as an example. It should be understood, however, that the following procedural descriptions may not be applicable to all institutions that utilize nonhuman primates because of differences in research needs, staffing, and facilities.

The quarantine facility should consist of animal rooms equipped with CDC requirements (Department of Health and Human Services, 2013). Prior to receipt of newly arrived monkeys, each cage should be washed in a cage washer and sanitized and the room washed, disinfected, and rinsed thoroughly. It is recommended that a product with tuberculocidal properties be utilized.

The monkeys are delivered in crates containing individually segregated animals. Shipment lots can contain up to 120 animals. Immediately after receipt into the institutional quarantine facility, the monkeys are transferred from their shipping containers to quarantine cages directly and with minimal handling. Drinking water, fruits and vegetables, and familiar foodstuffs are offered to assist in adaptation.

The animals are fed a commercial 15%–20% protein monkey diet supplemented with fruit on the afternoon of arrival and allowed to acclimatize to the laboratory conditions for a few days. After 3–5 days of acclimatization, the monkeys are sedated and examined. Stress to the animals and hazards associated with physical restraint are contraindicated; therefore, mild chemical restraint is a preferred method of handling for the animal's welfare, personnel safety, and CDC compliance. An electronic thermometer with an acrylic, rectal probe is used to obtain temperatures. The probe is shielded with a disposable plastic cover, which is changed and discarded between animals. Elevated body temperature in the sedated primate is a more accurate disease indicator than rectal temperature measured in the physically restrained animal as the animal's excitement level has a minimal effect of the core body temperature sedated. If there are clinical signs of diarrhea or blood in the stool, fecal cultures may be obtained prior to temperature measurement and must be handled according to the CDC requirements regarding samples exiting a quarantine room. It is recommended that the animals are physically examined by a veterinarian. All organ systems should be examined and results documented accordingly, including the evaluation of the ears, eyes, nose, oral cavity, lymph nodes, and cardiovascular, respiratory, abdominal, urogenital, musculoskeletal, and integument systems.

Minor health problems and any animals requiring supportive therapy treatment can be initiated at this time. Consideration of severe disease problems in individual monkeys that may require extensive clinical evaluation is reserved until all animals have been examined and released from CDC quarantine. Additional fecal cultures and hematological evaluations are performed as indicated. During physical examination, monkeys are tested for tuberculosis (TB) using 0.1 mL of veterinary mammalian tuberculin, full strength, administered intradermally in the eyelid. Each monkey is given an injection of ivermectin® the first week of the conditioning program. Additional parenteral anthelmintics (e.g., Droncit® for tapeworms and levamisole for roundworms) may be administered during the conditioning program as prescribed by the medication utilized and the conditioning plan of the facility. The second treatments with anthelmintics are administered 2 weeks following the initial administration during the conditioning program.

Prophylactic injections of amoxicillin (20–40 mg/kg subcutaneously) or enrofloxacin (5 mg/kg, intramuscularly) may be indicated for respiratory or gastrointestinal (GI) diseases, depending on the suspected infectious agent. Vitamins C and B complex (0.25–0.50 mL, IM) may also be given to improve the overall nutritional status of the animal, but the need should be identified and prescribed by the facility veterinarian.

Monkeys are tattooed consecutively, typically using a combination of letters and numbers per institution policy. Information such as weight, abnormalities, and clinical signs is entered on the animal's individual record.

The quarantine period procedures are based on the requirements of the CDC (Department of Health and Human Services, 2013).

Monkeys are quarantined for at least 31 days in order to diagnose and eradicate TB, but total time animals are quarantined averages approximately 42 days to include acclimation time and minor schedule modifications. A total of three consecutive, negative TB tests on each animal (after receipt) must be achieved prior to releasing the group from quarantine room. All monkeys that test positive for TB ("reactors") are examined, possibly radiographed, euthanized, necropsied, and tissued (CDC required the following tissues to be collected—liver, spleen, kidney, lungs, heart, tracheal bronchial lymph nodes, any gross lesions). Any gross lesions and tracheal bronchial lymph nodes are to be evaluated microscopically (acid-fast stain) for the presence of the

TB organisms, using PCR analysis, and cultured if acid-fast stain or PCR is positive. The CDC must be notified within 24 hours if an animal in quarantine has a positive result. If deemed to be positive for TB, the quarantine procedures for the remaining animals need to be reevaluated with notification and concurrence of CDC to determine if depopulation or a repeat of the quarantine period is appropriate.

All monkeys in the facility are observed daily by a technician or veterinarian. If needed, the veterinarian prescribes treatments and institutes diagnostic protocols for sick or debilitated animals. Urgency of treatment is paramount in any primate facility. For example, a debilitated animal exhibiting anorexia, nasal discharge, or diarrhea requires prompt attention. Treatment is initiated immediately to alleviate clinical signs. Diagnostic tests will only be performed if absolutely necessary during the CDC quarantine period.

Diarrhea is one of the most common clinical signs in monkeys, particularly in new or stressful environments. Diarrhea is initially treated symptomatically with an antidiarrheal such as pink bismuth (5 mL/kg/day, PO), high-fiber diet, probiotics, any combination, or all. Fecal collections for floatation and direct examination are collected to identify any parasites or protozoan present. Direct examination of feces may also identify blood and inflammatory cells indicative of infections. Rectal cultures are collected when blood or mucus is seen in the feces or multiple animals in the same room or group housing area, and a broad-spectrum antibiotic is administered. This original treatment is continued or changed depending on the results of culture and sensitivity testing. Other antidiarrheal agents, which have been used successfully, are aminopentamide hydrogen sulfate (0.1 mg/4.5 kg/day, intramuscularly), kanamycin sulfate® (10 mg/kg/day, intramuscularly), and sulfa drug combinations. Because specific animal groups usually harbor bacteria with similar antibiotic sensitivities, one drug can usually be employed for treatment for cohorts. Antibiotics are continued for the effective prescription time and/or at least 2 days after cessation of clinical signs. Fluid therapy (Lactated Ringer's® solution intravenously/subcutaneously or a commercially available oral electrolyte solution) is often used for monkeys with diarrhea and secondary dehydration. It is recommended to identify electrolyte and acid/base imbalances via serum chemistry analysis to effectively and efficiently treat the secondary diarrhea.

Bacterial pneumonia is occasionally associated with the stress of shipment. Supportive therapy, including fluids, nutritional support, a temperature-controlled environment, and administration of antibiotics (broad spectrum and/or high respiratory distribution, such as azithromycin), is recommended to treat pneumonia caused by secondary bacterial invaders. Severe epizootics of pneumonia have occurred in rhesus or cynomolgus monkeys that were associated with measles (rubeola) outbreaks the first few weeks of quarantine (Potkay et al., 1971). Vaccination with modified live measles vaccine is recommended at the supply facility prior to importation.

Other disease problems include amebiasis, dermatophytosis, frostbite (airline transportation), and miscellaneous traumatic injuries. Most of these infections or clinical conditions manifest themselves during the first 14–21 days after arrival at the facility. Thereafter, the health status of most animals stabilizes considerably during this time.

Blood samples are obtained following the quarantine period from animals to further evaluate their health status and for reference serology. Fecal examinations for intestinal parasites are conducted, using floatation and direct smear methods. Specific anthelmintics, antiprotozoal agents, and antibiotics are prescribed as indicated to individual monkeys or to groups.

The foregoing description of quarantine and conditioning procedures is applicable to cynomolgus and other macaques imported from purpose breeding farms (NAS, 1981; Renquist, 1975). Domestically bred macaques that have been tested and found to be free of infectious diseases communicable to humans (e.g., shigellosis, herpes B virus infection, and TB) do not require such rigid quarantine measures. It may be necessary, however, to provide a sufficient period of time for such monkeys to become adapted to a laboratory cage environment, since depending on their domestic supplier, the majority are born and raised in large harem-type cages or corrals. During the adaptation

period, valuable baseline data (hematological, serological, microbiological, physiological, and behavioral) may be compiled for individual monkeys.

Occupational Health Program

Transmission of disease between primates and humans has been described. Cases of herpes B infection are typically latent in the macaque, but usually fatal in humans, and have been incriminated in several deaths of animal handlers. An occupational health program is essential, including preemployment screens for TB, measles titer or vaccination, and physical examination with complete medical history (Huff and Barry, 2003). The occupational health program should also provide consistent periodic screens for TB, respiratory fit testing, and general physical health. Banked reference serum samples and continuous monitoring are necessary with evaluation by the physician to assure employee health. The reader is referred to the current National Institute for Occupational Safety and Health (NIOSH) standards for specific information on worker surveillance and evaluation criteria.

There are three central components to a sound occupational health program in a primate facility. The first component is the staff. They must be well trained in the biohazards and zoonotic diseases associated when working with primates. The institution must provide adequate personal protective equipment (PPE). Procedures and policies requiring the use of PPE should be incorporated into the training program.

The second component is to require written programs and procedures to be followed in the event that there is an exposure (scratch, bite, bodily fluid) from a primate or primate-soiled equipment. These procedures may include postexposure disinfecting of the wound or area, obtaining biological samples from the animal and human for surveillance, and providing medical care where appropriate.

The third component is the need to educate the medical community. Occupational health providers, emergency room staff, and medical specialists (e.g., virologist, trauma surgeons) should be a part of your medical support structure in the event that an exposure results in human disease. This will facilitate better care for your employees.

Common Diseases

The diseases and descriptions noted herein are those most common to the primate toxicology laboratory. A more comprehensive overview of the pathology associated with infectious diseases is presented in the pathology portion of this chapter.

Respiratory Diseases

Pneumonia is seen in primate colonies and is most often due to changes in environment related to stress of shipment or sudden changes in humidity or temperature. In macaques, this is often a complication of rubeola (measles) and specific bacterial pathogens, e.g., *Moraxella catarrhalis* or *Streptococcus* spp. Appropriate antibiotics during illness and preventative measures such as measles vaccination, quality housing, and decreasing stress are recommended (Good and May, 1971).

Enteric Diseases

Diarrhea and/or dysentery is probably the most common disease in nonhuman primates. The major causative organisms are gram-negative enteric bacteria (e.g., *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*, and *Escherichia coli*; refer to Weil et al., 1971 and Renquist, 1987).

Unfortunately, latent infection without clinical signs is common and can be exacerbated by the stress of the experimental protocol. Clearing protocols may be necessary to reduce the latent carrier state in infected animals; however, these antibiotic regimens are often costly and should not be performed without culture and sensitivity testing. Culture and sensitivity testing represents a single analysis of an evolving GI environment and the results reflect accordingly. When evidence of clinical disease is seen, appropriate therapy is often the best approach.

Tuberculosis

The bane of the research laboratory is TB caused by a variety of mycobacterial organisms (Renquist, 1987a). Appropriate tuberculin testing coupled with rigorous quarantine procedures can decrease the likelihood that it will enter the colony. A diligent intradermal mammalian tuberculin testing program, a health surveillance program to include radiology where appropriate, and specialized assays such as the interferon gamma test can be employed to achieve a comprehensive approach to TB prevention.

Viral Diseases

Measles (rubeola) is a common viral disease in humans and nonhuman primates. Certainly other viral pathogens, e.g., poxviruses, hepatitis, simian hemorrhagic fever, and rabies, have also caused problems in a variety of research facilities. Most recently, serious concerns have been caused by herpes B (Renquist, 1987b). The incidence of herpes B antibody is more prevalent in imported colonies, while domestic colonies are typically managed to be herpes B virus–free. Herpes B is latent in macaques, causing a perioral blister-like lesion that is typically not associated with other abnormalities. When a person is scratched or bitten by a positive primate, the individual must undergo monitoring for transmission of disease and have fatal consequences. The CDC has developed a set of guidelines for herpes B exposure and Ebola-like disease that should be carefully followed for both liability and employee health aspects (CDC Morbidity Mortality Report, 1987, 1990).

Simian retroviruses (SRVs) have been associated with morbidity that has confounded the results of toxicological studies. SRV is prevalent in many macaque populations and therefore should be part of a prestudy viral testing protocol. It is prudent to eliminate SRV-positive animals, both serology and PCR tested, from assignment to long-term and pivotal toxicological studies.

Licensing and Records

Licensing

The Animal Welfare Act requires the registration and inspection of primate facilities. The USDA Animal and Plant Health Inspection Service (APHIS) is involved with inspections and enforcement. Direct importers of primates must also be registered with the CDC.

Other regulations and agencies covering primates and their care and use are the Department of the Interior, Federal Wildlife Permit Regulations, Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and the National Academy of Sciences, Institute of Laboratory Animal Resources (ILAR).

Records

Accurate record keeping is required by law and is essential from the source to the completion of project. This involves the source country, date of receipt, importer, age, sex, body weight,

health histories, and tattoo numbers. This is particularly critical for animals for which a federal wildlife permit is required or when animals are on the threatened or endangered Convention on International Trade in Endangered Species list (e.g., chimpanzees).

Study Design

General Considerations

Proper design of the protocol for a preclinical toxicology study (also referred to as a study plan) is often the first and one of the most essential steps in safety assessment of a novel pharmaceutical agent, chemical, or toxin. The specific elements of the protocol are influenced by the intended therapeutic indication of an investigational drug and the patient (human or animal) population for which a drug is intended (Hobson and Fuller, 1987), or the potential route and level of exposure to a chemical or toxin for determining an appropriate risk assessment. To assist the researcher in protocol development, guidance documents issued by worldwide regulatory agencies, including the U.S. Food and Drug Administration (FDA), the U.S. Environmental Protection Agency (EPA), the European Union Organisation for Economic Cooperation and Development (OECD), the Japanese Guidelines for Nonclinical Studies of Drugs Manual, and the International Conference on Harmonization (ICH) Tripartite Guidelines, provide information on design of studies intended for safety assessment of drugs, chemicals, or toxins (referred to hereafter as test article).

The number of animals required for each study is directly impacted by the objectives of the study, the intended or expected duration of exposure, toxicologic endpoints, and potential need for assessment of posttreatment recovery. While true for any species used for research, considering the increased sensitivity for the use of nonhuman primates, the numbers of animals used in a study or program requires a balance of meeting study objectives with minimizing animal use (The Guide, 2011). Recommendations for the exact numbers of animals within a study group or study are not within the scope of guidance documents. In general, the number of nonhuman primates used in a study should be the minimum required for valid research results; thus it is incumbent upon the researcher to make sound scientific judgments on the appropriate number of animals and realistic justifications.

Similar to other research species, study duration for nonhuman primates ranges from single-dose acute studies to chronic multiple-dose studies that may exceed one year. Acute (single-dose) and short-term repeated dose toxicity studies are typically used to determine appropriate dose levels for subsequent studies of longer duration.

Selection of a Primate Species for Toxicology Studies

As discussed earlier in this chapter, there are many different species of nonhuman primates that can be used in research, and determining the best species for a particular study or program is challenging. An investigator should be aware that there may be more than one species suitable for toxicity testing. When choosing a nonhuman primate species for a toxicology program, it is often preferable to use one species, and from the same country or region of origin, throughout the program in order to minimize variability in clinical parameters (e.g., hematology and serum chemistry) and, to the extent possible, minimize genetic differences among the study animals.

In toxicological studies involving novel pharmaceutical candidates, animal availability and industry trends have had a significant impact on the choice of the nonhuman primate species. From the early research into polio vaccine development (the 1940s) and continuing until approximately 25 years ago, the rhesus macaque was the species of choice for most pharmaceutical development programs. Today the cynomolgus macaque is the most common nonhuman primate species used in toxicology studies. Cynomolgus monkeys have some advantages such as a smaller body size with

young adults in the range of 2–8 kg (Bonadio, 2000). Dedicated breeding facilities provide defined populations of animals with known health status and fewer limitations on availability (McCann, 2007). The rhesus monkey remains a suitable (although infrequently used) option from a scientific standpoint, and specific programs may be the best choice. The greater prevalence of biotechnology-derived pharmaceuticals has further increased the need to consider the exact genetics of specific species, as some agents may not have equal activity across the nonhuman primate species. Other considerations when choosing a nonhuman primate species for studies include study design, dose administration techniques, health specifications such as specific pathogen-free animals, body weight and body size of the animal, and measures of overall maturity (i.e., sexual, skeletal, and neurological development).

Humane Endpoints

Humane endpoints are the physical, clinical, or other in-life observations that mark or describe adverse toxicity that can result in an animal welfare concern. These warrant close evaluation as in-life data may serve as early predictive indicators of present or impending adverse effects. If excessive or of sufficient severity, occurrences of pain, distress, or an illness as a direct result of the study procedures may progress beyond acceptable humane levels. Close monitoring allows for these endpoints to be used to effectively modify the procedures or, if necessary, euthanize the animals prior to mortality and potential loss of valuable data.

As noted, humane endpoints in nonhuman primates can be an effective tool; however, consistency among animal care staff, veterinarians, investigator/study directors, and the IACUC (when needed) is essential. Humane endpoints are intended to describe or quantitate the type, course, and progression of adverse toxicities. As such, the types of endpoints and how each is measured should be determined by active communication between all members of the study team prior to study initiation. Furthermore, it is critical for any humane endpoint determination that individuals performing the assessment are qualified in the evaluation of these parameters, and consistent methodology is employed across the study personnel and throughout the study duration.

Dose Levels

Dose levels tested are usually based on a proposed clinical (human) dose or estimates of human exposure. The lowest dosage in a safety study is usually equal to or greater than the expected human dose and should define the no-effect level (NOEL) or no-adverse-effect level (NOAEL). The highest dose is some multiple (e.g., 10–100×) of the proposed clinical dose and is expected to induce toxicity. The middose is an intermediate level, usually with minimal toxicity. When considered across the dose range, the data should characterize a dose–response relationship between the test article, exposure values, and measurements and observations of toxicity.

Dosing Techniques

Dosing procedures used in research studies will vary depending upon the type of program, experimental objectives, or the biochemical characteristics of the test article. When possible, the dosing technique utilized in safety studies for drug candidates with nonhuman primates approximates as closely as feasible the expected route and frequency in humans. For test articles intended to be taken by patients as a tablet, capsule, or other oral format, safety studies in nonhuman primates are typically conducted using nasogastric intubation, oral gavage, and instillation of a capsule or by masking in dietary supplements. On occasion, a test article may be administered using alternate oral routes such as in fruit slices, sugar cubes, or a fruit juice drink. Biotechnology-derived test articles are most often given intravenously (injection or intravenous [IV] infusion) or by subcutaneous

injection and occasionally by intramuscular injection. Less common routes of dose administration are intranasal, intraperitoneal, intraocular, or site-directed administration, e.g., intrajejunal, via the hepatic artery, or intra-articular. Methods of dosing may also be dictated by the volume needed to achieve the intended dose levels, characteristics of formulation of the test article such as pH and viscosity, and the duration of administration.

For all dose routes, procedures for dose administration should minimize stress to the animal, yet protect personnel and animals from injury. Infectious agents that may be carried by some species of nonhuman primate (e.g., herpes B virus) may be transmitted to animal handlers with potentially fatal consequences. The fecal–oral transmission of bacterial pathogens (*Salmonella*, *Shigella*, *Campylobacter*, and *E. coli*, to name a few) can potentially occur, leading to disease in the animal handler. As a result, the zoonotic potential of these and other infectious agents must be considered for all handling and dosing procedures. However, with proper training of personnel in the handling of nonhuman primates, these potential can minimize the risks and studies involving multiple-dose routes and procedures successfully completed.

Dose Volumes

Guidelines for maximum dose volume administration vary from facility to facility, and there is a paucity of information available in the literature. Researchers are encouraged to check with their facility veterinarians and/or IACUC or similar groups within the facility in which the studies will be conducted. Suggested maximal dosing volumes for nonhuman primates are described in Table 9.2.

Dose Preparation/Formulation

Preparation/formulation is typically conducted on the day of dosing, although there may be instances where the stability of the test article is sufficiently known such that it allows for preparation of dosing material at an earlier date. A number of vehicles are used for formulation of an oral liquid, depending on the aqueous solubility of the test article. True solutions, where aqueous solubility is high, commonly use distilled water, simple or buffered saline, or aqueous admixtures that increase stability and solubility. Polyethylene glycol (PEG400, or similar) is commonly used when the aqueous solubility of the test article is poor; however, dose volumes should not exceed 1 mL/kg due to the increased incidence of diarrhea with this vehicle. Suspensions of the test article are commonly prepared in carboxymethylcellulose (typically 1%), and this material is well tolerated in nonhuman primates. Capsules can be prepared prior to the day of use, if a powder; if liquid, it is necessary to fill the capsule soon before administration. Control animals should be administered a concentration and volume of the vehicle equivalent to that received by the high-dose animals.

Table 9.2 Maximum Single Dose Administration Limits

Route	Maximum Volume	Comments
Capsule	Single "0" capsule	Capsule size limitation due to anatomic limitations.
Nasogastric/oral	10 mL/kg	Per dose; twice daily dosing possible, minimum 4 h apart.
Intramuscular	0.5 mL/kg	Per dose; outer thigh or triceps in larger animals.
Subcutaneous	5 mL/kg	Per dose; dorsal midscapular area.
IV bolus ($\leq$3 min)	10 mL/kg	Also described as "slow push" or injection.
IV infusion ($\leq$1 h)	15 mL/kg	Maximum volume may be exceeded depending on formulation.
IV infusion (1–6 h)	10 mL/kg/h	Animal should be closely monitored for urine output.
IV infusion (6–24 h)	5 mL/kg/h	Single dose; monitor urine output.
IV infusion (>24 h)	4 mL/kg/h	Continuous infusion, chronic administration possible.

Preparation of Solutions for Injections or Infusions

For oral or IV administration, the individual doses are drawn up into graduated syringes in the formulation laboratory or in the animal room. If prepared in a formulation laboratory, syringes are labeled for clear identification, providing the animal number, the study, and group color code. This information is verified by a second technician not involved in the dose preparation. Syringes are placed in a rack, which may be labeled or color-coded, and are transported to the animal room. If dose withdrawal occurs in an animal room, the source container is appropriately identified with the study and group information. Dose volume for each animal and dose administration is verified and documented by the technical staff conducting the dosing. Depending on the study design, animals may be fasted overnight prior to a morning dosing. Caustic or irritating compounds or large gavage volumes may induce an emetic response that, if aspirated in the lungs, can result in the death of the animal. Consideration must be given to reducing the dose or reformulating the test article to reduce the possibility of aspiration.

Oral Administration

Oral administration of drugs in safety studies has generally been conducted in squirrel monkeys or young adult/adult macaques and in young or small baboons. The reason for size limitations is the expense of special handling equipment and the size and aggressive nature of mature monkeys and great apes.

Techniques for Oral Administration

Techniques for oral administration of a test article include nasogastric gavage, oral gavage, capsule (or other solid form) using a modified gavage tube, or, when palatable, putting the drug on a piece of fruit or mixing in a drink such as fruit juice.

Equipment for Oral Administration

The nasogastric route of administration is the most common and preferable means of gavage administration for young adult to adult cynomolgus and rhesus monkeys. Although larger monkeys may be manually restrained for oral dosing procedures, the risk of injury to personnel is high, as the head of the animal must be held securely by animal technicians.

Commercially available infant feeding tubes are best suited for nasogastric intubation. An 8 French (ID) feeding tube is generally (Figure 9.5) used with small volumes or low-viscosity liquids. When oral gavage is preferred, either due to the size of the animal, restraint method used, or test article properties, commercial gavage tubes in size from 8 to 12 French (ID) can be used. Specialized tubing is required for dosing capsules or other solid forms. Tygon or polyethylene tubing of appropriate size can be custom modified with one end of the tube beveled and then blunted by heat to prevent esophageal damage or stomach injury. For nasogastric dosing and orogastric administration of liquids or capsules, a syringe is attached to the tube for delivery of the encapsulated test article and/or flushing the tube.

Manual restraint can be used for dosing of smaller species, e.g., squirrel monkeys or young cynomolgus or rhesus monkeys. A chair restraint system may be needed for larger monkeys. This method restrains the animal's head and limbs during the dose administration procedure, minimizing the risk to technical personnel. In addition, this restraint system can be less stressful to the animal than manual restraint, if the animal is properly acclimated to the procedure. When using a chair restraint system, an aluminum or hard plastic collar is placed around the neck of an anesthetized

Figure 9.5 Nasogastric dose administration.

animal and remains in place for the duration of the study. Aluminum poles with snap-type hooks are inserted into the cage and attached to the collars. The animal is led from the cage and placed in a chair (Figure 9.5) and the collar secured to the chair. In this manner, minimal handling of the animal's head is required for dosing. A technician, wearing appropriate gloves and arm covers, restrains the animal's head, the tube is inserted, and slight negative pressure is applied to the syringe to check for air bubbles, indicating a possible lung intubation. The dose is administered, a flushing syringe containing a small amount of water or vehicle is then attached, and the remaining contents of the tube are flushed into the stomach. The tube is removed and the animal is observed for a moment to check for vomiting. The poles are reattached to the collar and the animal returned to their cage.

Risks to the animals are minimal if proper procedure is followed; however, if the dose is inadvertently placed in the lung rather than the stomach, chances for animal survival are minimal. Animals typically adapt to this handling procedure within a short period of time (typically 1–2 weeks) (Figure 9.6).

Capsule (Oral Gavage) Administration

Plain gelatin or enteric-coated capsules are administered when testing a potential formulation system or when a slower absorption rate is required. The technique for dosing is similar to oral dosing with a liquid. The plain or coated capsule is lightly wedged into the beveled end of an 8 to 14 in. length of tubing of slightly larger inside diameter than the diameter of the capsule. A syringe, usually containing 10–15 mL of tap water, is attached to the other end of the tube. The capsule-plugged end of the tube is intubated and the capsule flushed into the stomach. The administration of multiple capsules simultaneously is possible, but this is generally used only in single-dose studies due to the difficulty of this procedure. Nevertheless, in appropriate several capsules can be administered without repeated intubations. A stylet may also be used to expel capsules out of the tube
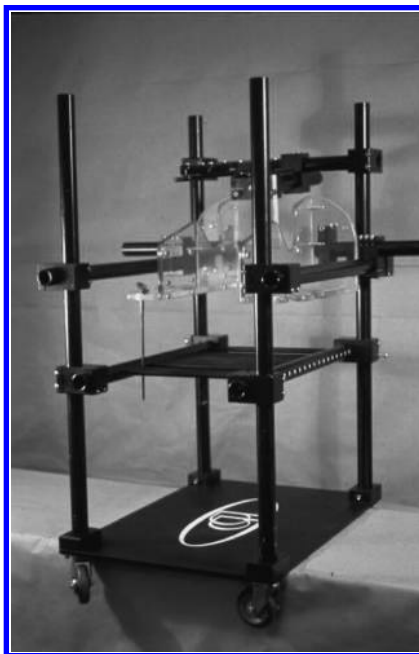
Figure 9.6   Primate restraining chair.

once properly placed in the stomach. Prestudy acclimation to the dosing procedures will minimize the possibility of dosing errors and animal injury. Capsule administration is not recommended for studies in nonhuman primates of longer duration (greater than 14 days of daily administration), due to the increasing chance of injury to the animal from the larger tube diameter.

Other Oral Dose Administrations

Administration via incorporation in food rations or insertion into edible treats can be a means of dosing. However, nonhuman primates have a propensity for manipulating their food and expelling portions of their dietary rations from their cage or breaking it up and dropping it through the cage floor, thus making it essentially impossible to quantify dietary consumption or assure a specific dose of test article. On a limited basis, small quantities of test article have been placed on sugar cubes, in small pieces of fruit, or dissolved in a commercially available fruit drink. Animal technicians will present the test article–containing food item and observe to verify that it is consumed by the animal. Liquid may be administered from a syringe such that the animal voluntarily drinks the dosing solution. Expected problems encountered with these methods include removal of portions of the sugar cube or fruit from their mouth and refusing to complete the dosing, or losing some of the fruit drink through spillage, and refusing to swallow all of their dose solution. These problems can be minimized if the test article/food mixture is highly palatable to the animal. Additionally, the effects of the content of such vehicles on absorption and metabolism of the test article must be ascertained as part of the study design.

Restraint Techniques

Manual restraint methods include cageside dosing, removal from the cage and immobilization by the technician, tube restraint, and chair restraint. Cageside dosing is achieved by using the

squeeze-back mechanism of the animal's cage, assuming this type of caging is being used. Once the animal is appropriately restrained, the dose is administered. This method can be accomplished by one technician. For dose administration outside of the cage, the animal is caught (using protective gear) after partial closing of the squeeze-back mechanism. The animal is removed from the cage and restrained, typically by cradling on the side/hip of the technician. A second technician administers the dose, which is confirmed by another individual in the room.

For tube restraint, which is most appropriate for IV dose administration and femoral venous blood collection, the same method of extracting the animal from the cage is used, and the animal is placed headfirst into an appropriately sized clear Lucite tube. The animal's legs are restrained and the dose is administered into the saphenous vein. For chair restraint dosing, two handlers, each with a pole, are required to capture an animal and placement in a primate chair. A third technician, meanwhile, has the animal's dosing syringe and tube (if nasogastric or oral) available for dose administration.

Parenteral Administration

The IV route of administration is a commonly employed technique for nonhuman primates in toxicology studies. This route is preferred for test articles with poor bioavailability and/or limited GI absorption and for biotechnology-derived proteins and monoclonal antibodies that are not appropriate for oral administration. The cephalic and saphenous veins are the most accessible for peripheral venipuncture, in both single- and repeat-dose studies. Peripheral veins, including the femoral and the jugular, can be catheterized for acute or subchronic IV infusions, although many veins require surgically invasive procedures for catheter placement. In addition, specialized restraint devices are required for protection of the catheters from the animals, e.g., primate chairs, vascular ports, jacket–tether infusion, or backpack-port ambulatory infusion systems. Intramuscular or subcutaneous injection can also be utilized for parenteral administration.

Peripheral Venous (Bolus) Administration

The two largest and most accessible superficial veins for IV dose administration are the cephalic and saphenous veins. The cephalic vein is located on the dorsal arm near the bend of the elbow. The saphenous vein can be found on the ventral side of the leg from the knee to the ankle. Toxicology studies involving single daily IV injection of bolus infusion for as long as 12 months can be performed successfully, if proper techniques are utilized to minimize vascular and perivascular tissue damage.

Syringe and needle sizes vary with the volume and characteristics of the test article to be injected. Needle sizes usually range from 21 to 25-gauge and syringes from 1 to 35 mL. Injection volumes greater than 10 mL are more successfully administered when a small butterfly catheter is placed into the vein for dosing. This may be facilitated by the use of two technicians, one to restrain the limb and ensure that the butterfly is not dislodged and the second to administer the dose through the distal end of the catheter and subsequently flush the remaining dose with appropriate vehicle (e.g., saline).

Alert Capture for Bolus Administration

Small- and medium-sized monkeys, up to 5 kg, can be caught and restrained by technicians wearing heavy leather gloves with long gauntlets, by reaching into the cage and securely grasping a limb and body and gently removing it from the cage. The animal is restrained on a table, stomach down, exposing the back of the leg for a saphenous injection. A second technician can then administer the dose. Large macaques and small baboons are usually not removed from their cages for dosing. In facilities utilizing squeeze cages as the home cage, the animal is pulled to the front of the cage by the squeeze mechanism, which is locked in a forward position. The technician carefully

Figure 9.7   IV (saphenous) injection of rhesus monkey using a squeeze-back device and cage restraint.

maneuvers an arm or leg near the feeding hole in the lower front of the cage. Once the foot or hand is gripped tightly, the squeeze mechanism is allowed to return approximately one-third of the width of the cage, permitting the animal to lean toward the rear of the cage. The limb can now be extended through the hole for accessibility to the vein (Figure 9.7). Prior to injection, a portable clipper is used to remove the hair from 2 to 4 in. along the length of the vein. The exposed area is cleaned with an alcohol pad and pressure applied to the vein with the thumb while holding the limb with the hand. The pressure applied proximal to the intended injection site impedes blood flow and the vein becomes visible to the technician. A needle, attached to the dosing syringe, is placed bevel up against the skin. Pressure is exerted until there is a slight give, usually indicating entry into the vein. Slight back pressure on the syringe will draw blood into the hub of the needle, verifying venous placement, at which point the test article may be administered.

Tube Restraint for Bolus Administration

The tube restraint technique is used for IV bolus (or slow push) administration into the saphenous vein. The tube is constructed of clear Lucite plastic and varies in wall thickness, diameter, and length. A variety of tube sizes should be available to accommodate differences in age, species, and size of monkeys to be restrained. Animals acclimate to the tube restraint technique quickly, as the barrier between the handler and animal not only provides a greater level of safety but also, with repeated use, has a calming effect on the animals. This restraint technique should also be considered when frequent blood sampling is required, as it minimizes the potential for inadvertent cageside trauma (i.e., bruising and abrasions) and has been found to decrease errors related to strict time collection requirements.

Cage Restraint Technique for Dosing, Sampling, and General Handling Procedures

An additional method of restraint has been developed utilizing a procedural cage that is mounted to the front of the cage (Figure 9.8). This device was developed to minimize the stress-related

Figure 9.8 Procedure cage mounted to the front of an individual cage in a standard two-by-two rolling rack.

changes in behavior, as well as changes in endocrine hormones and clinical hematology and chemistry parameters, and to provide a safe, efficient, and effective means to execute dosing, sampling, and body weight collection procedures. The procedure cage is mounted to the front of the home cage and the door is opened. Using the squeeze back of the home cage, the animal is encouraged to move into the procedure cage and the door is closed. Once inside, the animal remains calm and various procedures can be performed. Since all procedures are performed outside the home cage, it remains a safe place and minimizes stress while enhancing the safety of animal handlers. Additional information with regard to the procedure cage can be provided by Shin Nippon Biomedical Laboratories, Ltd. (SNBL).

Intravenous Infusion

A variety of techniques are utilized to administer dosing solutions via slow IV infusion. The following section provides a brief overview of these techniques; however, a more comprehensive discussion of IV infusion in nonhuman primates can be found in the *Handbook of Pre-Clinical Intravenous Infusion* (Ed. Healing and Smith, 2000, second edition in preparation).

Chair Restraint Technique for Infusion

Studies requiring an IV infusion for periods of time ranging from 30 min to (a suggested) maximum of 4 hours can be conducted using primate restraint chairs. Studies requiring a longer duration of infusion, or repeated daily doses similar to the maximum infusion duration, should be conducted using the jacket–tether or ambulatory infusion model (see later).

For chaired infusions, animals should be adapted to chair restraint prior to study initiation. This is best completed with several chairing sessions beginning two weeks prior to the start of the study to reduce stress related to the chairing procedure. Depending on facility guidelines, the preparation may be in conscious animals or with brief anesthesia in which animals are first anesthetized with

ketamine HCl (approximately 10 mg/kg). An indwelling catheter is introduced into the cephalic or saphenous vein (Flynn and Guilboud, 1988). Provision must be made to secure the hands and arms to prevent the catheter from being pulled from the vein by the animal's free hand(s). The prepared dose is drawn up in an appropriate-sized syringe and placed on an infusion pump set to deliver the designated dose volume at a preset rate. An extension tube (typically 10 in. in length) is attached from the syringe to the catheter for delivery of the dosing solution. The volume of the extension tube (and other external components, such as in-line filters) must be factored into the dose preparation calculations to assure sufficient volumes to dose all animals. At the termination of dosing, the catheter is removed, homeostasis is induced, and the animal is removed from the chair and returned to the home cage.

Jacket and Tether Technique for Infusion

The femoral and jugular veins are ideal for chronic catheterization and the conduct of chronic infusion studies. Externalization of an indwelling catheter involves a surgical procedure, in which the catheter is channeled subcutaneously to an exit site located on the dorsal thoracic region of the animal, and it is protected by a jacket and tether system (Bryant, 1980). This offers the advantage of limiting the animal's accessibility to the catheter.

Animals are first acclimated to the tethers and jackets for 2–3 days prior to placement of the catheter. During this period, the jacket is worn by the animal while the tether (flexible steel tube) hangs freely in the cage unattached to the animal. After the acclimatization period, the animal is sedated and taken to the surgery suite. The animal is prepared for surgery and an incision made in the skin above the vein and/or artery to be catheterized. The vessel is located and separated from connective tissue by blunt dissection. The vessel is elevated with sutures to impede blood flow and a small cut is made in the vessel with iris scissors. A catheter introducer is inserted into the incision to facilitate introduction of the catheter. The catheter is inserted into the vein or artery for 5–8 in., depending upon the size of the animal. The most desirable placement of the catheter is in the femoral artery or vein so that the catheter tip extends to either the vena cava or aorta, facilitating infusion and/or blood sampling procedures. The catheter is held in place by ligatures placed around the vessel and on both sides of an elevation (or "donut") located several inches above the catheter. This prevents the catheter from being pulled from the vein or artery by pressure exerted from normal body movement. As an additional precaution, a stress loop is made in the catheter and anchored with a suture(s) just under the skin. A trocar is used to tunnel the catheter under the skin to the middle of the back where it is exited. The catheter is then passed through the mounting plate attached to the jacket and through the tether to the appropriate channel on the underside of the swivel. Another length of catheter is attached to the same channel on the top of the swivel and then to an infusion pump. Aseptic techniques in handling the exposed end of the catheter are extremely important to the success of this system.

Following a short recovery period, the dosing formulation is delivered via an infusion pump external to the cage, and doses can be administered over extended periods of time, i.e., several hours or days. These procedures are applicable for new- and old-world monkeys, but are not feasible for animals as large as adult chimpanzees. Frequent observations for potential problems with jackets and the tethering system are important if the study objectives are to be met (Figure 9.9). Primates are particularly adept at manipulating this system and thus require frequent monitoring to correct any attempts by the animal to remove the jacket.

VAP Administration

The vascular access port (VAP) is a subcutaneously implanted device that provides chronic vascular access and eliminates the need for multiple venipunctures in repeat-dose IV bolus or

Figure 9.9 Cynomolgus monkey fitted with ambulatory infusion jacket. The winged infusion set is used to access the VAP, which is implanted in the dorsolateral thoracic region under the jacket.

intravenous infusion studies (Swindle et al., 2005). The VAP is a silicone rubber septum housed in a titanium metal casing that can be anchored with sutures under the skin. The placement of the vascular port is usually in the dorsolateral thoracic region, from which the catheter, typically polyurethane or silicone, is tunneled under the skin to the femoral vein (or another vein/artery) for catheterization. Dosing is performed by injecting the test article into the port and subsequently the catheterized vein.

VAPs have proven to be quite durable and reliable, as long as local and systemic infection is rigorously monitored and treated proactively. Repeat-dose studies in excess of nine months in duration have been successfully conducted; however, it should be recognized that continuous use of these devices results in an increasing rate of catheter failure after 13 weeks of dosing. Catheter failure is typically preceded by repeated dermal and/or subcutaneous infections, formation of adhesions, and necrosis of the skin overlying the VAP, as well as infections along the catheter tract characterized by swelling and occasional necrosis/drainage. While there is no clear explanation for the increased incidence of catheter failure in chronic studies, gross and histopathologic findings from animals chronically instrumented with VAPs indicate that changes occur at the catheterization site. At gross necropsy, tissue adjacent and distal to the tip of the catheter thickens and becomes more brittle, and strictures form as fibrous connective tissue, replacing the nonviable regional vasculature over time. Histopathologic findings include fibrosis of the vascular wall, fibrous capsule formation, chronic active inflammation, thrombosis, and hypertrophy of vascular/perivascular tissues (Swindle et al., 2005). Location of the port and septum is easily accomplished by palpation. The area of the skin above the port is prepared using aseptic techniques, to minimize the chance of infection. To prevent a "coring effect," a 21-gauge Huber-point (noncoring) needle is used for port access. If possible, locking solutions (often containing heparin or other anticoagulants) should be aspirated prior to flushing the port. When flushing the port and administering a dosing formulation, the Huber needle is inserted through the septum, and the dose is administered. A saline (or saline-containing heparin

up to 40 IU/mL) solution is used to flush the remaining test article from the catheter, and the appropriate locking solution is instilled to maintain catheter patency.

Ambulatory Intravenous Infusion via VAP

Ambulatory infusion employs the use of a VAP for dose administration and offers some advantages over the jacket and tether technique. Animals are first instrumented with a VAP, and sufficient healing time (approximately 7–10 days) is allowed prior to initiation of acclimation procedures. Each animal is fitted with an appropriately sized infusion jacket, which contains a dorsal opaque (black canvas) pocket. A battery-powered ambulatory infusion pump (CADD® Legacy PLUS, SIMS Deltec, Inc., Minneapolis, MN, or equivalent) and self-contained dosing reservoir (50 or 100 mL cassette fill volume), along with a winged infusion set (i.e., infusion line connecting the pump/reservoir to the VAP via Huber-point needle), are contained within the dorsal pocket (Figures 9.9 and 9.10).

The ambulatory infusion system allows the investigator to implant a number of animals prior to initiation of dosing, thus providing a readily available pool of animals for a series of studies. If intermittent infusion is required (i.e., multiple daily doses or a defined dosing intervals per week), this system can result in decreased animal handling due to the programmable nature of the infusion pump. In addition, the jacket and infusion system may be removed between doses (such as once or twice weekly), allowing the animal more normal activities and minimizing infusion system-related injuries, i.e., pressure necrosis to the skin, obscured infections, etc.

Ambulatory infusion is not appropriate for test articles with limited room temperature stability, or for those that require very small or very large volumes of administration, i.e., less than 5 mL/dose or over 250 mL/dose for the designated infusion period. In addition, this system is not optimal for animals larger than 5 kg, due to handling and safety concerns. Additional information

Figure 9.10 Cynomolgus monkey moving freely in the home cage using the ambulatory infusion system.

concerning the use of ambulatory infusion in nonhuman primates can be found in the earlier referenced literature as well as the bibliography for this chapter.

Intramuscular Injection

Intramuscular administration provides for a reasonably consistent means for a rapid absorption and distribution profile, depending upon the test article characteristics and the properties of the vehicle/excipients. Biologics delivered in oil are released much slower than from an aqueous carrier; in addition, suspensions, where the particle size is larger than for true solutions, may have a longer time to maximal plasma concentration (C_{max}) and a concomitant longer half-life ($t_{1/2}$). In nonhuman primates, the injection site is usually the outer thigh (quadriceps femoris mm.), although the triceps are also utilized in larger animals. Dosing volume should be limited to less than 0.5 mL/kg to minimize pain and the incidence of skeletal muscle irritation. If larger dosing volumes are required, two injection sites should be utilized.

Moderate-sized cynomolgus and rhesus monkeys and small baboons can be dosed alert, whereas large baboons and chimpanzees should be sedated. For alert dosing, a cage squeeze mechanism allows the animal to be transiently immobilized to allow access for injection to a leg or arm. Hair is clipped from the injection site and the site is either cleansed with alcohol or aseptically prepared using a Betadine solution. Using a syringe and appropriate-sized needle, usually no larger than 21-gauge, the skin and muscle are penetrated. A slight vacuum is applied by the plunger to check for possible venous puncture. If blood is drawn into the syringe, the needle should be removed and the process repeated. If no blood appears, the plunger is depressed slowly until the dose is administered. The needle is then removed and pressure applied to the injection site for approximately 30 s to complete the dosing procedure.

Subcutaneous Injection

Subcutaneous administration is frequently utilized in toxicity studies when a slower absorption rate is required or if the characteristics of the formulation, i.e., low pH, large-particle suspension, large volume, etc., preclude the use of the intramuscular route. Subcutaneous injection is also a preferred route for self-administration by patients treated with biotechnology-derived pharmaceuticals making this route directly applicable to clinical and commercial use.

In nonhuman primates, the dose is delivered between the dermal and muscle layers, usually in the dorsal midscapular area. The loose skin at this site allows for volumes up to 5 mL/kg to be administered. Other subcutaneous injection sites to be considered are the inner thigh and limbs for smaller volumes of dosing formulation, i.e., less than 0.5 mL/kg. One animal technician should restrain the animal while another administers the dose, to ensure complete delivery of the test article and prevent possible injury to the animal or handler.

Capture and restraint procedures for the animals are as previously described. The injection site is shaved and cleansed with alcohol or prepared aseptically with a Betadine solution. The skin is grasped between the thumb and forefinger and retracted from the underlying muscle. The skin is penetrated with the needle at approximately a 15° angle to the injection site. As the plunger is depressed, a small bubble should appear as the dosing progresses. There should be no resistance to the injection. If resistance is noted and the bubble is firm, close to the surface, and appears white in color, then the injection is being administered intradermally and replacement of the needle is required. The needle should be inserted to the deepest point into the subcutaneous space initially, slowly removing the needle as dosing is completed to assure a broad distribution of the dosing formulation. The needle exit site should be compressed for approximately 30 s after dosing to prevent leakage of the formulation. Prior to the injection, the relatively large potential subcutaneous space at

each dose site should be demarcated with either a tattoo or indelible pen, depending on the duration of dosing and need to evaluate the dose site(s) for adverse reactions.

Miscellaneous Routes of Administration

Intranasal

The development of aerosol delivery systems for intranasal administration has resulted in increased numbers of intranasal toxicity assessments. Test articles are administered into one (or both) nostrils, often with the aid of a calibrated and metered aerosolizer device. Discharge from the reservoir can be accurately calibrated, but total delivery into the nasal cavity is not always successful (or consistent) in an alert animal. Depending on the frequency of dosing and size of the animal, dosing can be accomplished via hand restraint (i.e., one animal handler for restraint and one technician for dose administration) or via the use of chair restraint (for multiple doses over a short period of time or for larger monkeys). This technique allows for better control of the head while the delivery device is placed a short distance into the nostril for dose administration.

Inhalation toxicology, for test articles intended to be delivered to the lungs, is a unique area requiring specialized equipment and facilities not usually found in general toxicology laboratories and is thus beyond the scope of this review.

Intraocular

Periocular and intraocular dosing, i.e., administration of dosing formulations around the eye or via intravitreal injection, is an increasingly common route for the development of targeted therapies for degenerative ocular disease. Nonhuman primates provide an excellent ophthalmology model because of the similarity in ocular anatomy and physiology to the human eye. Similar to humans and unlike other laboratory species, nonhuman primates have a macula and do not have a tapetum lucidum. Test article can be administered to nonhuman primates via several dose routes including topical to the eye, intravitreal injection, and subconjunctival injection.

Standard ocular evaluations in toxicology studies include ophthalmoscopic and biomicroscopic (slit-lamp) examination. More specialized evaluation of retinal vascularity is conducted by fluorescein angiography, intraocular pressure may be measured by pneumotonometry, and retinal conductance is evaluated by electroretinography (ERG).

Currently, research efforts are targeted to many serious ocular diseases such as glaucoma, diabetic retinopathy, and macular degeneration. A commonly used model in the macaque uses a laser to induce retinal neovascularization (a response by the choroid to the laser lesion) to mimic neovascularization associated with macular degeneration. This model may be used to evaluate potential drugs targeted to preventing or treating neovascularization (Criswell et al., 2004; Shen et al., 2004).

Intraperitoneal

Although not a common route for administration to nonhuman primates, intraperitoneal injections or infusions permit rapid absorption into the portal circulation. In cancer patients, this route would place therapeutic agents in close proximity to tumors of the abdominal organs. This method has been utilized successfully in nonhuman primates in safety assessment studies for delivery of tumor-specific monoclonal antibodies.

After anesthetization, the entire abdomen and inner upper thigh are shaved, and the entire area and more specifically the injection site, which is 1–2 in. below and 1–2 in. lateral to the navel, are cleaned using a Betadine swab or solution (or equivalent). The skin adjacent to the injection site is pulled slightly to the center of the abdomen with the thumb of one hand while inserting the needle at

an angle of approximately 45° into the injection site with the other hand. The purpose for pulling the skin to one side and placing the needle or catheter at an angle ensures that when the needle/catheter is removed, an interrupted channel into the body cavity is established. This reduces bleeding and decreases the chances for infection. A short catheter (2 in.) or needle reduces the chances of perforating the bladder, intestine, or other organs. The location of the needle/catheter can be checked by injecting 1–2 mL of saline followed by gentle vacuum with a syringe. If blood, fecal material, urine, or fluid other than saline is aspirated into the syringe, the procedure must be reattempted on the opposite side. Health-threatening consequences could result from organ perforation; thus a veterinarian should be notified immediately and appropriate antibiotic therapy begun if this occurs or is suspected.

Upon successful insertion, thumb pressure on the skin is released, which will stabilize the needle or catheter at the site. As the plunger of the syringe is depressed, attention must be directed at the injection site for possible swelling, which would indicate a subcutaneous or intramuscular dose. If swelling occurs, dosing is stopped and the procedure repeated. Following completion of the injection, the needle/catheter is removed and pressure applied to the injection site for approximately 30 s followed by close observation for any bleeding or swelling at the site.

Repeated interperitoneal injections (needle or catheter) to nonhuman primates are not recommended because of the high probability of infection as well as the potential for bladder or intestinal puncture.

Infrequent Routes of Dose Administration

Drug delivery systems designed for special therapeutic applications have been evaluated in safety studies but are not considered "routine" procedures. Such procedures include osmotic mini pumps or other drug-containing implants, topical administration; vaginal, rectal, and/or colonic administration via suppositories or liquids; and sublingual dosing. Many of these unique routes of dose administration require surgical procedures or can only be administered to anesthetized animals or animals physically restrained in primate chairs. Such methods are typically utilized only for acute dosing protocols and may not be acceptable for subchronic or chronic toxicity assessments.

Data and Sample Collection Techniques

All nonhuman primate toxicology studies intended to be in compliance with GLP regulations require approved protocols, including a defined listing of in-life and postlife evaluations to adequately characterize and evaluate the toxicological and pharmacological effects of the test article. As noted previously, various regulatory agencies provide guidance as to the basic elements of regulatory toxicology study designs, but much is left to the individual researcher to define and justify the specific parameters to be evaluated. Commonly used evaluations include daily clinical (cageside) observations, body weights, blood collection for serum chemistry, hematology and coagulation, and urinalysis evaluations. Additional endpoints may include physical examinations, measurement of cardiovascular parameters, ophthalmic examinations (direct or indirect), and serum/plasma test article concentrations for determination of a toxicokinetic profile. Occasionally, more specialized tests or samples such as telemetry-based cardiopulmonary monitoring, nerve conduction velocity, electroencephalograms (EEGs), ERGs, and tissue biopsy are required. After completion of the dosing phase, animals are commonly euthanized, gross necropsies are conducted, organ weights are measured, and a protocol-specified list of tissues is collected, preserved, processed, and evaluated for potential histopathologic changes. Most studies of 4 weeks' duration or longer include both terminal phase (i.e., immediately after completion of dosing) and recovery phase groups of animals to assess the potential for the reversal of target organ changes and/or toxicity. Recovery phases range from 2 weeks to a number of months, depending on factors including the known toxicology, pharmacology, and tissue distribution and toxicokinetics of the test article.

Both study design and animal inventory planning must consider that nonhuman primates should only be subjected to one major experimental surgical procedure during their lifetime (Federal Register, 1991). A major surgical procedure is defined as one that penetrates a body cavity or is considered invasive in nature; examples include bone surgery, intracranial administration of test article, or medical device implantation. Additional procedures within the same experimental protocol must be approved by the testing facility IACUC, include appropriate perioperative measures for analgesia, and can in some cases be justified by decreasing the overall number of animals used in experimental research.

Clinical Observations

Because nonhuman primates have complex and highly individualized behavior patterns, obtaining meaningful observation data depends on the experience of the observer, the knowledge of expected observations for the species in a laboratory setting, and an innate familiarity with individual animal responses. The use of group housing (gang caging) makes this task even more complex for toxicologic evaluations; however, behavior studies prefer the establishment and maintenance of a well-defined animal hierarchy (Bramblet, 1994).

Ideally, clinical observations should be made at the same time(s) each day and by the same observer each day. Many studies include postdose evaluations that coincide with the expected maximum plasma concentrations (C_{max}), which allows the researcher to determine if any effects are present after dose administration and/or recover prior to the next scheduled dose. Changes in behavior or appearance are most important to note. An experienced observer should be quick to note (Silverman, 1988):

- Lethargy
- Dehydration
- Unusual posture or movement within the cage
- Poor condition of hair coat
- Brightness or dullness of the eyes
- Unusual fluid from body orifices
- Unusual motion within the cage and/or proprioceptive deficits
- Self-mutilation
- Hyperventilation
- Hyperactivity
- Hyperreactivity (startle responses)
- Apparent pain or discomfort
- "Favoring" of individual limbs
- Evidence of changes in appetite or water consumption (if possible)
- Watery or discolored stool
- Changes at injection sites (where relevant)
- Any other unusual behavior or appearance

Appetite should be assessed at least once daily and is commonly conducted prior to offering the morning food ration. Persistent inappetance can result in serious, and possibly life-threatening, hypoglycemia in most nonhuman primate species, as animals have limited stores of glycogen and become ketotic with starvation.

Physical Examinations

Physical examination findings can be one of the most important overall criteria to determine acceptability of an animal for study use. These evaluations include, at a minimum, the assessment of

the overall physical condition of the animal, body temperature, respiration rate, heart rate, thoracic auscultation, abdominal palpation, integumentary assessment, overall behavior and mentation, and other evaluations that are specified in the testing facility SOPs and/or study protocol. Specialized examinations, which can include endocrinological profiles or more extensive neurological examinations, may be required, depending on the type of toxicology study (Keeling and Wolf, 1975).

Clinical pathology (serum chemistry, hematology, and coagulation parameters) and fecal parasite evaluations are typically included in the general physical examination. Tables 9.5 and 9.6 detail typical normative clinical hematology and serum chemistry data for nonhuman primates commonly used in toxicity evaluation studies.

Body Weights

Toxicologists who are not accustomed to reviewing data from nonhuman primate studies are often surprised by the degree of inter- and intra-animal variability in body weights. For example, body weights for study populations that include male and female young adult/adult cynomolgus monkeys can range from 2 to 8 kg at study assignment. It is not uncommon in a study of 2 weeks' duration to encounter individual weight fluctuations of 10% or more that are unrelated to direct actions by the test article. Rather, these fluctuations can be the result of daily study activities that impact normal animal behavior, e.g., blood draws, dosing procedures, or transient fasting for blood draws or dosing. Study procedure–induced changes can be minimized by weighing and feeding at the same time each day, when appropriate acclimating the animals to in-life procedures prior to initiation of dosing and providing individualized care to animals that may not adapt as quickly to the requirements of the study. Longer-term acclimatization to the room, the diet, and the other animals in the room is also advantageous for some types of studies (Renquist, 1975).

Anesthetized animals can be weighed on conventional scales; however, the use of transfer box, tared to provide a net body weight, allows for data collection without the use of a sedating or immobilizing agent. Animals can be readily trained to enter the boxes, particularly if they are housed in cages with squeeze-back mechanisms.

Physiological Measurements

Physiological endpoint measurements have become routine in safety evaluations, and are used to determine if acute effects occur that have an adverse effect on the cardiorespiratory and central (CNS)/peripheral nervous system. Historically, the requirement for such measurements resulted from observations of pyrogenic activity of certain proteins and cardiotoxic or neurotoxic effects of monoclonal antibodies. Indications of untoward physiological responses in the past have necessitated a thorough evaluation of these parameters. Comprehensive batteries of physiological measurements, except neurological examinations, are usually obtained under ketamine immobilization to allow for a more thorough evaluation of all organ and body systems; however, some measurements may be collected in an alert animal due to the sequence of study-related activities and the need to monitor the animals during the postdose period(s).

Body Temperature (Rectal)

Rectal temperatures are obtained using a digital thermometer coated with lubricant (K-Y Jelly® or equivalent). The animal is placed in the prone position on a table or gurney for rectal accessibility. The probe, covered with a lubricated disposable plastic sheath, is gently inserted into the rectum and remains in place until a body temperature reading is attained. Rectal temperatures should be recorded as quickly as possible after sedation for the most accurate reading. Rectal temperature

may be obtained in alert animals using manual restraint, but excitement due to the procedure results in wide variations in temperature compared to measurements obtained from lightly sedated animals.

Blood Pressure

It should be noted that the most accurate data for blood pressures are obtained through using telemetry and surgically implanted sensors, but such techniques have the disadvantage of requiring surgical implantation of a pressure transducer into the femoral artery. As a result, with the exception of studies that include radiotelemetry monitoring in subgroups of animals, most toxicology studies use alternative means for obtaining systolic, diastolic, and mean arterial blood pressure. Pressures are obtained indirectly using a blood pressure machine, in which the cuff is designed for small animals or infants. The blood pressure cuff is placed securely around the upper thigh or forearm with the cuff-positioning arrow situated over the medial midfemoral or humeral region. The cuff may also be placed around the base of the tail. The machine is started and the reading on the gauge is recorded. The blood pressure assessment should be repeated at least two additional times and a mean calculated to ensure an accurate estimate. Blood pressure readings using the cuff method may be affected by movement of the animal, so it is critical that the animal remain as quiet as possible during the recording.

Heart Rate

The standard method for recording the heart rate requires using a stethoscope to hear the heartbeat. Using a stopwatch, the heart rate is counted over a minimum period of 15 s and the appropriate multiplication factor applied. The heart rate can also be determined by calculations from ECGs, with blood pressure monitors equipped to record this measurement, or when telemetry units are used for blood pressure collection.

Respiration Rate

Respiration rates can be obtained cageside by observing the breathing pattern of the animal. If the nonhuman primate was recently removed from the cage for dose administration or other study-related procedures, sufficient time should be factored into the procedure to allow the respiration rate to return to expected resting values. Calculations from counting over a set period of time are made similar to those used for heart rate data.

Neurological Evaluation

A neurological examination is a series of subjective observations conducted to evaluate both central and peripheral sensory and motor functions. These evaluations are performed by veterinarians or research personnel experienced in observing nonhuman primates in a laboratory environment. A freely moving animal is initially examined cageside, followed by removal from the cage and assessment of specific neurologic pathways via reflex testing. An example of a grading system is as follows (Table 9.3).

Additional tests require the animal to be drawn to the front of the cage (if using squeeze-type caging) or properly restrained outside the cage. Pelvic or thoracic limb flexor reflexes can be assessed by exerting pressure with a hemostat (or similar) to the lateral and medial digits. The integrity of the reflex arc following mild pressure stimulus is graded as responsive (+) or nonresponsive (−).

Table 9.3   Grading Scale for Effect on Mobility

Grade	Sign
0	Absence of purposeful movement—paraplegia
1	Unable to stand to support; slight movement when supported by the tail—severe paraparesis
2	Unable to stand to support; when assisted, moves limbs readily but stumbles and falls frequently—moderate paraparesis and ataxia
3	Can stand to support but frequently stumbles and falls—mild paraparesis and ataxia
4	Can stand to support—minimal paraparesis and ataxia
5	Normal strength and coordination

Table 9.4   Grading Scale for Patellar Reflex

Grade	Sign
0	No reflex
1	Hyporeflexic
2	Normal
3	Hyperreflexic
4	Clonic/tonic

Patellar reflexes (femoral nerves) are evaluated while the animal is as relaxed as possible. The patellar tendon is lightly tapped with a reflex hammer and subjectively graded as follows (Table 9.4).

Cranial nerves are evaluated by observing a variety of reflexes and positional responses, which allows the examiner to determine if the test article has a CNS effect that can be localized to specific region(s) of the brain. Among these evaluations are pupillary reflexes, assessment of pupillary size (both relative and degree of dilation/restriction), the absence or presence of involuntary ocular movements (nystagmus), improper positioning of one or both eyes (strabismus), the presence or absence of the menace reflex, and responses to controlled auditory stimuli. Additional tests can be conducted depending on the expected pharmacologic or toxicologic action of the test article.

Ophthalmologic Examinations

Ophthalmologic examinations are now routine in toxicology studies regardless of the route of administration, due to a heightened concern for potential ocular toxicity. The eye is a complex organ that includes functional, neurological, and vascular components that can be highly sensitive to potential insult by test articles. Ophthalmologic examinations commonly occur prior to initiation of dosing and then again prior to scheduled necropsies or release from study to determine treatment-related effects. Documentation of the presence of preexisting abnormalities is critical to determine if changes observed at later timepoints are related to the test article.

The ophthalmic examination involves, at a minimum, the assessment of the health of the ocular adnexa, e.g., eyelids, conjunctiva, external surface of the cornea, sclera, and associated surface vasculature. These examinations can be conducted on alert animals using a restraint chair. However, if the examination requires an assessment of the anterior and posterior chambers and associated structures, the animal is best chemically restrained with ketamine. Prior to the examination, a few drops of a topical mydriatic agent are placed in the surface of the eye to achieve papillary dilation. When immobilized, the ocular adnexa are thoroughly evaluated. A fundic examination is performed using a direct or an indirect ophthalmoscope, allowing the veterinarian (or other trained professional) to observe the anterior chamber, iris, posterior chamber (vitreous), fundus, optic disc, macula, and the choroidal structures.

Electrocardiograms

ECGs measure the electrical conduction activity of the heart and are a standardized assessment of cardiac function and health. Examinations should occur prior to initiation of dosing and at one or more timepoints during the dosing and recovery periods (if included in the study design). Given the potentially transient nature of changes to cardiac electrical activity associated with test article pharmacology/toxicity, ECGs should be collected predose and at a defined timepoint postdose, typically at the expected time of maximum test article concentration (C_{max}) in blood or tissues.

ECGs are collected on alert animals using a Plexiglas® restraint board. The animal is removed from the cage and manually restrained; assuming animal size allows for adequate protection to the animal and technician. In general, the animal is positioned on its back in a horizontal position. The hair is shaved from each site designated for the placement of an ECG lead or electrode. After shaving, the site is wiped with alcohol and the appropriate ECG lead is attached to the skin using an alligator-style clip. The leads are attached to the following sites to provide a standard 6-lead (I, II, III, aVR, aVL, and aVF) ECG recording (Table 9.5).

Both qualitative (e.g., assessment of tracings for the presence of abnormal complexes) and quantitative (e.g., measurement of specific intervals) data can be derived manually or electronically, depending on the collection and assessment methods. The recent discovery of test article–induced changes in cardiac function and the potential for cardiac arrest has led to an increased emphasis on quantitative ECG assessments. The determination of QT intervals, corrected for heart rate (QTc), has become increasingly common when the pharmacology of a test article suggests a potential effect on the heart. There are various formulas used to correct the QT interval, and they are available in the current literature for review and consideration. While quantitative data can be determined in the context of a toxicology study, specific and more detailed evaluation may be best obtained in a dedicated cardiovascular safety pharmacology study.

Invasive Cardiovascular Procedures

Direct monitoring of central arterial and venous pressure is the most common cardiovascular measurement requiring an invasive procedure. Normally, animals are surgically catheterized in the femoral vein or artery, connected to a direct recording device, and monitored for up to several hours under general anesthesia. IV infusion of ketamine or an inhalant anesthetic such as isoflurane can be used.

Table 9.5   Placement of ECG Leads

Labeled Leads	Lead Placement/Location
Left arm (LA)	Wrist of the left arm
Right arm (RA)	Wrist of the right arm
Left leg (LL)	Calf of the left leg
Right leg (RL)	Calf of the right leg
V1	4th intercostal space, approximately 4 cm from midsternal line
V2	4th intercostal space, approximately 4 cm from midsternal line (i.e., on the left side of symmetrical MV1)
V3	Left midaxillary line in the 5th intercostal space (approximately 1 cm below level of MV2)
V4	7th thoracic vertebra

Measurement of cardiac output is possible with the thermal dilution technique in large cynomolgus or rhesus monkeys or smaller baboons using human pediatric catheters. Smaller animals are not suitable for this technique because appropriate catheters are not available at this time. Microspheres can be used to estimate cardiac output in smaller animals (Forsyth et al., 1968). Normal cardiovascular values have been reviewed by Bourne (1975). Similar to that noted earlier, invasive cardiovascular parameters may be best obtained in a dedicated cardiovascular safety pharmacology study.

Blood Collection and Normative Clinical Pathology Data

Trained and experienced technicians can collect blood samples from alert (unanesthetized) nonhuman primates or young baboons in most situations without danger to either the animal or the technician. If the technicians are not experienced, if the animals are large, if the cages are not the appropriate type, or if the blood volume needed is large, then animals should be anesthetized prior to venipuncture. Some studies have shown physiological and immunological changes caused by behavioral stress with alert bleeds (Mason, 1972). These possibilities must be considered in any study design. In certain instances, macaques, baboons, and chimpanzees can be trained to submit voluntarily to blood collection procedures (Hein et al., 1989; Vertein and Reinhardt, 1989); however, routine blood collections in the larger species are almost always done under anesthesia (Muchmore, 1973) because of the danger of injury to technicians by stronger animals and increased activity during blood sampling from alert animals can adversely affect some clinical parameters (e.g., will elevate creatine phosphokinase and corticosteroids; refer to Coe et al., 1985).

Blood samples are commonly collected by direct venipuncture. Occasionally, samples are collected through temporary indwelling catheters when frequent collections are required, as in PK studies. Rarely, samples are collected through long-term indwelling catheters. In general, venipuncture is preferred because of less damage caused to the veins, less need for anesthesia or minor surgery, and a reduced chance of infections. In some instances, samples have been collected several times daily for up to a month by venipuncture without adverse effects if aseptic techniques are used (shave and aseptic preparation of the collection site).

Venipuncture samples requiring small amounts (less than 5 mL) may be collected from the cephalic or saphenous veins. The femoral vein is generally used when blood volumes greater than 5 mL are required. For cephalic or saphenous vein collections, animals are restrained as described in the previous sections. The skin over the vein is shaved and swabbed with alcohol or an iodine solution. Either a syringe and needle or an evacuated container can be used. The needle should be the smallest size compatible with the sample volume, i.e., 23-gauge for samples less than 1 mL, 22-gauge for l to 3 mL samples, etc. Smaller needles cause less damage to the vein and lessen the hazard of hematomas forming. Short-bevel needles are preferred when they are available because they have less chance of slicing the vein wall. After withdrawal of the needle, direct pressure should be maintained on the site for approximately 30 s. If femoral samples are collected from anesthetized animals, pressure should be maintained on the site for at least 1 min to minimize the incidence of hematoma formation.

The amount of blood that can safely be removed from a nonhuman primate during the course of a study depends on the study purpose, the study length, total blood volume (Bender, 1955), and the frequency and amount of samples (Keeling and Wolf, 1975). Total blood volume for cynomolgus or rhesus monkey is estimated to be approximately 6% of body weight, i.e., 325 and 280 mL for a 5 kg cynomolgus or rhesus, respectively (Diehl et al., 2001). Recommended and maximum volumes for blood collection vary considerably among laboratories and institutions. As a general guideline, 10%–15% of the blood volume can be removed every 2–4 weeks (Diehl et al., 2001). Larger volumes collected less frequently may have a different impact as compared to smaller volumes collected more frequently. If an important endpoint of the study is a hematological evaluation, then the

maximum recommended volume should be conservatively interpreted in order to avoid influencing these endpoints. When in doubt, hematological parameters should be monitored frequently to avoid adverse effects.

Some laboratories return red cells to the animal in an attempt to increase the maximum amount of blood that can be collected. In general, this technique should be avoided, as the risk of infection and hematological changes outweigh the benefits gained.

Temporary catheters placed in restrained or anesthetized animals provide a means for collecting very frequent samples over a short period of time. They are introduced by the technique described earlier for IV infusion (also see Bowen and Cummins, 1985). Chronic catheterization may be an option for repeated blood collection; however, this can provide a route for serious infections even when the most careful aseptic techniques are used. Moreover, chronically placed catheters frequently develop fibrin flaps over their distal end, effectively blocking blood withdrawal. When catheters are used, patency can be improved by filling the catheter volume with a normal saline solution containing 1–4 IU/mL sodium heparin for injection. Regular flushing of the catheter and strict aseptic procedures are required to maintain patency in a chronic catheter for periods longer than a week. Tables 9.6 and 9.7 summarize normal ranges for hematology and serum chemistry laboratory values in the five most common nonhuman primate species.

Urine Collection

Nonhuman primates have a tendency to manipulate their food and water (or watering devices), as such quantitative timed collections of uncontaminated urine or feces are a difficult task (Baker and Morris, 1979). By placing a collecting pan (with screens optional) under the cage and removing the food and water source, short-term urine samples of reasonable quality can be obtained from a single-void specimen. Samples should be collected early in the morning, placing the pans beneath the cage prior to the start of light cycle for the room. When the daily light and activity cycle begins, most animals void quickly. When setting up urine collection cages for metabolism studies or for studies that require urinary electrolyte excretion studies, space is provided under or adjacent to the collection pans to place a collection vessel in ice. This is important when 24 hours total urine collections are required. Inexpensive, Styrofoam coolers can be modified and are quite useful for holding the ice and urine collection vessel and also have the added benefit of protecting the resultant sample from ambient light.

Specialized PK studies may require quantitative urine collection over a limited period of time. This is frequently accomplished by placing the animal in a chair and catheterizing the bladder of females and placing a condom collection device over the penis of males (bladder catheterization is not practical as a routine, alert procedure in males). If animals are appropriately adapted to the chair, this technique can be used for up to 4 hours. Longer periods of chairing are best interrupted with an exercise period to minimize stress-related changes. Other methods used in a chemically restrained animal include a direct sample collection by suprapubic puncture (Keeling and Wolf, 1975) or an indirect collection by manual bladder expression. Neither method is recommended for repeated and/or frequent sample collections.

Pharmacokinetic and Toxicokinetic Evaluations

PK/toxicokinetic studies involve the collection of serial blood samples to assess the overall absorption, metabolism, and elimination kinetics of a test article. Samples are collected to provide an adequate quantity of the appropriate matrix (i.e., serum or plasma) as well as a sufficient number of timepoints to allow for calculation of pertinent data. Toxicokinetics involve the mathematical and statistical manipulation of the data to determine the behavior of the test article in the blood or other biologic matrix.

Table 9.6 Hematology of Nonhuman Primates, Normal Ranges

Test	Units	Squirrel monkey[c,h] (*Saimiri sciureus*)	Cynomolgus monkey[b,d] (*Macaca fascicularis*)	Rhesus monkey[a,d] (*Macaca mulatta*)	Baboon[d,e] (*Papio* sp.)	Chimpanzee[d,f,g] (*Pan troglodytes*)
White blood cells (WBC)	thsd/mm^3	3.4–14.8	4.5–14.0	5.5–15.4	—	7.2–16.8
RBCs	million/mm^3	7.1–10.9	4.6–6.5	4.0–6.6	—	4.8–5.8
Hgb	g%	12.9–17.0	10.0–13.1	10.9–15.5	8.7–13.9	12.0–5.8
Hematocrit (HCT)	%	—	30–41	35.2–47.6	31–43	36.0–49.5
Mean corpuscular volume (MCV)	µm^3	41.4–62.7	57–68	65–79	63–90	64–102
Mean corpuscular hemoglobin (MCH)	µµg	13.9–20.1	18–22	21–27	18–27	20–34
Mean corpuscular hemoglobin concentration (MCHC)	%	29.2–34.8	30–33	30–34	28–34	27–36
Platelets (PLT)	thsd/mm^3	—	200–550	230–650	225–544	150–450
Reticulocytes	%	—	0.1–1.1	0–1.9	0.3–2.3	0.5–1.5
Differential						
Segmented neutrophils (SEGS)	%	13.0–79.0	Oct-50	32–83	23–78	55–80
Lymphocytes (LYMPH)	%	19.0–82.0	50–80	34–69	14–76	Dec-45
Monocytes (MONO)	%	0.0–6.0	0–2	0–2	0–3	0–5
Eosinophils (EOS)	%	0.0–22.0	8-Feb	0–6	0–8	0–2
Basophils (BASO)	%	0.0–4.0	0–2	0–1	0–1	0–1
Coagulation:						
Prothrombin time (PT)	s	—	8.9–12.9	9.4–13.4	11.5–13.0	10.0–13.4
Activated partial thromboplastin time (APTT)	s	—	16.9–33.9	19.5–25.3	29.5–38.0	18.9–35.6
Fibrin degradation products (FDP)	µg%	—	<8	<8	—	<8
Fibrinogen (FIBRIN)	mg%	—	150–300	187–411	180–230	139–416

[a] Carpenter, J. W., Characterisation of the rhesus monkey CYP3A64 enzyme species comparison, 3rd edn. *Exotic Animal Formulary*, 2004.

[b] Lee, R. and Doane, C. J., *APV Primate Formulary*, 2003.

[c] Hainsey, B. M. et al., (Pan Andrade et al.; Goncalves, Miguel Angelo Bruck; Marques, Marco Antonio Pereira; Cabello, Pedro Hernan; Leite, Jose Paulo Galiardi. Mem Inst. Oswaldo Cruz, Rio de Janeiro, 99(6), 581, October 2004.

[d] Fortman, J. D. et al., *The Laboratory Nonhuman Primate Guide* 2002, Taylor, CRC Press.

[e] Soto, V. M. D. et al., *Contemp. Top. Lab. Anim. Sci.*, 44(1), 29, January 2005.

[f] Sackett, G. P. and Elias, K. eds., *Nursery Rearing of Nonhuman Primates in the 21st Century*. Springer, 2006.

[g] Holly Smith, B. et al., *Am. J. Phys. Anthropol.*, 37(S19), 177, 1994.

[h] Baldonado, R. P. Q. et al., *Jose Paulo Galiardi Mem. Int. Oswaldo Cruz*, *Rio de Janerio*, 98, October 2003.

Table 9.7   Blood Chemistry of Nonhuman Primates, Normal Ranges

Test	Units	Squirrel Monkey[c,h] (*Saimiri sciureus*)	Cynomolgus Monkey[b,d] (*Macaca fascicularis*)	Rhesus Monkey[a,d] (*Macaca mulatta*)	Baboon[d,e] (*Papio* sp.)	Chimpanzee[d,f,g] (*Pan troglodytes*)
ALT	U/L	4–1612	May-50	May-61	—	0–40
Albumin (ALB)	mg%	—	3.4–5.0	3.6–5.5	3.6–5.5	2.7–5.3
Alkaline phosphatase (ALP)	U/L	6.0–49.0	150–464	45–661	154–1105	142–624
AST	U/L	—	May-50	Dec-63	—	0–40
Bicarbonate (BICARB)	mmL/L	—	20–32	96–116	—	25–32
Bilirubin, total (TBILI)	mg%	0.0–1.9	0.1–0.8	0.1–1.2	0.3–0.7	0.1–1.3
Blood urea nitrogen (BUN)	mg%	11.4–42.2	28-Aug	8.38	25-Sep	22-May
Calcium (CA)	mg%	4.2–5.8	8.4–11.1	8.1–13.8	8.0–9.6	8.3–12.9
Chloride (CL)	mmL/L	103.0–118.0	100–118	16–29	104–118	94–110
Cholesterol (CHOL)	mg%	116.0–272.0	70–178	62–238	68–232	144–304
Creatine phosphokinase (CPK)	U/L	—	206–630	22–53	—	Sep-33
Creatinine (CREAT)	mg%	—	0.5–1.2	0.1–1.5	0.8–1.4	0.1–1.3
Gamma-glutamyltransferase (GGT)	U/L	—	Nov-50	24–645	—	42–366
Globulin (GLOB)	mg%	—	1.5–4.8	1.5–4.8	2.4–4.4	1.9–4.9
Glucose (CLUC)	mg%	35.0—148.0	48.0–80	27—100	50–129	75–117
Lactic dehydrogenase (LDH)	U/L	230.0–760.0	100–446	43–426	99–488	105–439
Phosphorus (PHOS)	mg%	3.2–9.2	2.1–5.5	2.4–8.7	4.7–7.7	3.0–6.4
Potassium (K)	mmL/L	3.5–9.5	3.5–5.0	2.93–5.45	3.2–4.3	3.5–5.3
Protein, total (TPROT)	mg%	—	6.2–7.6	5.9–8.8	5.7–7.8	5.5–7.2
Sodium (Na)	mmL/L	144.0–173.0	135–158	135–158	143–158	133–148

[a] Carpenter, J. W., Characterisation of the rhesus monkey CYP3A64 enzyme species comparison, 3rd edn. *Exotic Animal Formulary*, 2004.
[b] Lee, R. and Doane, C. J., *APV Primate Formulary*, 2003.
[c] Hainsey, B. M. et al., (Pan Andrade et al.; Goncalves, Miguel Angelo Bruck; Marques, Marco Antonio Pereira; Cabello, Pedro Hernan; Leite, Jose Paulo Galiardi. Mem Inst. Oswaldo Cruz, Rio de Janeiro, 99(6), 581, October 2004.
[d] Fortman, J. D. et al., *The Laboratory Nonhuman Primate Guide* 2002, Taylor & Francis, CRC Press.
[e] Soto, V. M. D. et al., *Contemp. Top. Lab. Anim. Sci.*, 44(1), 29, January 2005.
[f] Sackett, G. P. and Elias, K. eds., *Nursery Rearing of Nonhuman Primates in the 21st Century*. Springer, 2006.
[g] Holly Smith, B. et al., *Am. J. Phys. Anthropol.*, 37(S19), 177, 1994.
[h] Baldonado, R. P. Q. et al., *Jose Paulo Galiardi Mem. Int. Oswaldo Cruz*, *Rio de Janerio*, 98, October 2003.

The following kinetic parameters are typically derived separately for males and females in all groups on the intervals (i.e., Day 1 and subsequent timepoints):

- C_{max} = maximum plasma/serum/matrix concentration
- t_{max} = time of maximum plasma/serum/matrix concentration
- t_{last} = time of last quantifiable plasma/serum/matrix concentration
- AUC_{last} = area under the concentration versus time curve from time zero to the time of the last quantifiable plasma/serum/matrix concentration
- $AUC_{0-\infty}$ = area under the concentration versus time curve from time zero to infinity
- $t_{1/2}$ = apparent terminal elimination half-life

These data provide a more integrated assessment of test article exposure, to include potential accumulation and gender effects, as it relates to clinical observations, clinical pathology, gross necropsy, and histopathologic findings. The reader is encouraged to consult a variety of references that more fully address this subject.

Necropsy

The determination if one or more tissues (or organ systems) were adversely affected by a test article is often critical for a comprehensive safety evaluation. The necropsy findings, both gross and histomorphologic, should focus on the antemortem and postmortem findings of the study and any associations with humane endpoints (i.e., clinical signs), body weights, physical exams, and clinical pathology. Gross necropsy and histological changes may also be essential in the determination of cause of death or morbidity of study animals.

Owing to the potential for transmission of zoonotic diseases from nonhuman primates to humans, it is essential that strict safety procedures be adhered to during necropsies. Personnel protection equipment include gloves (two layers for prosectors), masks, face shields, disposable surgical gowns, and other specialized protective wear as required for the individual study and as designated by facility SOPs.

It is essential that a prenecropsy meeting be held with prosectors, pathologists, and study directors to familiarize all involved with the protocol, previous antemortem findings, etc. This will enable any recorded abnormalities (noted at the time of clinical observations or physical examinations) to be noted for potential correlation at necropsy.

Although necropsy procedures may vary slightly depending on the facility, species evaluated, and study objectives, the basic steps are relatively standard. The study protocol should be reviewed in detail to determine the specific requirements, organ weights to be measured, tissues collected, and any unique procedures, such as frozen tissues, whole-body perfusion, or unconventional tissue collection and preservation procedures. The testing facility SOPs detail the most common procedures, and the study protocol is referenced to detail any unique requirements.

Specialty Pharmacology/Toxicology

Safety Pharmacology

Safety pharmacology studies are designed to evaluate the potential for test articles to produce secondary pharmacological or toxicological effects within specific organ systems that are acute and potentially life threatening. As described in the S7A ICH guidelines (ICH, 2000), these types of studies were developed to protect clinical trial participants and patients receiving marketed products from potential adverse effects of pharmaceuticals. In some cases, studies in nonhuman primates provide the best means of assessing the toxicity of prospective pharmaceuticals on physiological functions in relation to exposure in the therapeutic range and at multiples of this range. These types of studies are intended to supplement existing information to satisfy international regulatory guidelines for non(pre)clinical safety evaluation that recommends investigation of potential ancillary pharmacologic actions (S7A: Safety Pharmacology Studies for Human Pharmaceuticals, International Conference on Harmonisation, July 2001 and S7B: Safety Pharmacology Studies for Assessing the Potential for Delayed Ventricular Repolarization [QT Interval Prolongation] by Human Pharmaceutics, International Conference on Harmonisation, May 2005).

The cardiovascular, respiratory, and central nervous systems are considered to be the vital organ systems whose functions are critical for life. According to the ICH guideline, these organ systems comprise the "core battery" that should be evaluated prior to first administration to human beings by appropriate dedicated safety pharmacology studies or included within toxicology studies.

Cardiovascular assessment is the most common core battery test evaluated in the nonhuman primate. The use of telemetry technology in unrestrained nonhuman primates provides a useful model to assess the primary effects of test articles on ECG and cardiovascular parameters. For the cardiovascular system, the S7A ICH guideline suggests measurement of blood pressure, heart rate, and ECG. Importantly, telemetry provides a greater opportunity for the detection of conduction abnormalities and adverse effects on repolarization by measurement of the QT interval. Test article–induced, heart rate–independent prolongation of the QT interval has been associated with torsades de pointes and fatal arrhythmias in humans (Yap et al., 2003).

As noted previously, blood pressure, heart rate, and ECG data can be collected without surgical intervention for telemetry unit placement. However, telemetry technology with implantable instrumentation offers advantages over models that require physical or chemical restraint (anesthetized model). The lack of restraint facilitates the collection of more physiologically meaningful data and increases the likelihood of detecting test article–related adverse effects on the cardiovascular system.

While less common, the integration of respiratory and CNS parameters into the design of the cardiovascular safety pharmacology study, toxicology study, or as-dedicated studies accommodates the assessment of the respiratory and central nervous systems and satisfies the ICH S7A guideline for adequately evaluating the "core battery." For the "respiratory system," the respiratory rate (via telemetry or visual assessment) in conjunction with measures of respiratory function, e.g., hemoglobin (Hgb) oxygen saturation or tidal volume, is generally sufficient. For the CNS, motor activity, behavioral changes, coordination, and sensory/motor reflex responses using functional observation battery (FOB) and body temperature (via telemetry or rectal) are generally sufficient.

Neurotoxicology

Animal models of neurotoxic insults are valuable to the extent that they provide a sensitive and valid index of the onset and/or progression of test article–induced deficits. For deficits that are similar across a wide range of species, such as axonal degeneration in peripheral nerves (e.g., length-dependant distal axonopathy), adequate models may be limited to rodents. In contrast, there are some neurotoxic deficits that affect portions of the neuraxis that are specifically vulnerable, have specialized anatomy, or involve transmitter distributions that are unique to primates. For these cases, in-life neurotoxicology assessments are best addressed in studies in nonhuman primates. For example, in macaques and humans the distal projection of bipolar neurons in the dorsal root ganglia (DRG) forms the distal peripheral nerve, while the central projection of many of these cells enters the spinal cord and forms the ascending dorsal columns without synapsing. Thus, axons in the spinal cord of nonhuman primates can represent the processes of first-order sensory neurons and be damaged by pathology limited to the DRG (e.g., buildup of heavy metals such as platinum). When considering a test article that may induce a central myelopathy (e.g., γ-vinyl GABA), the volume of the subcortical white matter makes these regions vulnerable in primates. An assessment of the potential for myelin-based damage should include evaluation of the major ascending tracts, such as the lateral lemniscus and the corpus callosum in a nonhuman primate model. In addition, for test articles that target specific neurotransmitters, such as those used to treat psychiatric disorders, nonhuman primates must be a part of the preclinical safety evaluation because of the primate-specific pattern and distribution of neurotransmitters, such as NMDA.

The neurotoxicity of a novel therapeutic drug is ultimately related to its PK, metabolism, and the potential for metabolite-induced neurotoxicity and the degree and speed of the parent or metabolite(s) to penetrate the blood–brain and blood–nerve barriers. The clinical impact of these elements is best modeled in nonhuman primates.

Developmental and Reproductive Toxicology

Nonhuman primates are an important model system for male and female fertility, juvenile toxicology, teratology, and peri- and postnatal studies, due to their phylogenetic, reproductive, and developmental similarities to humans (Hendrickx and Cukierski, 1987; Hendricx and Dukelow, 1995). Treatment of women of childbearing age, e.g., birth control products, preterm labor, vaccines taken during pregnancy, or used for autoimmune diseases, or men prior to and/or during conception, e.g., potential for direct actions on spermatogenesis or transferred in semen, is a relatively common practice. Furthermore, many biotechnology-derived drugs are highly target-specific immunomodulators and may only cross-react with nonhuman primates, and as such testing in rodents would not address the potential impacts of pharmacology. Therefore, the need for developmental and reproductive safety assessments performed in nonhuman primates have increased.

Treatment dose and regimen and study length are dependent on the potential use of the test article in humans. There are regulatory considerations that impact study designs and address both the development of small molecules and biologics (CDER, 2011). The cynomolgus monkey is the most frequently used nonhuman primates in reproductive toxicity testing, and the following discussion is limited to this species, but will mention the rhesus as necessary (given the significant body of research in this species and the occasional use in safety evaluation studies today). To model the use of drugs intended for the reproductively active population, this sometimes requires a three-segment testing plan, not to be confused with the standard three-segment reproductive evaluation in rodents (Hendrickx and Binkerd, 1990; Hendrickx and Cukierski, 1987). In segment I, now commonly included in general safety assessment studies of 3 months or longer (ICH, 2011), menstrual cycles and occasionally hormone levels are monitored for 90–180 days, depending on protocol design, and testicular volume, spermatogenesis, and rarely testosterone levels are evaluated in males during a minimum 90-day treatment period. In segment II studies, now commonly included in pre-/postnatal studies or enhancing pre-/postnatal studies (ePPND), pregnant females are treated during the period of organogenesis (gestation days 21–89), and a cesarean section is usually performed on day 100 or 140 for assessment of fetal abnormalities. The late gestational effects of a test material are examined in segment III studies, now commonly included in pre-/postnatal studies or enhancing pre- or postnatal studies, and ePPND designs. Pregnant females are administered with the drug from gestation days 90–150 for evaluation of any abnormal neonatal neurological or behavioral responses.

Reproductive toxicology studies in nonhuman primates are labor intensive because the menstrual cycle of each individual animal must be tracked to determine ovulation for the optimal mating days. As compared to cynomolgus monkeys, rhesus monkeys are used less frequently due to seasonal infertility. When rhesus is the more appropriate species, a timed breeding model has been developed using exogenous progesterone to induce an artificial luteal phase for studies that require dosing of test article during the early gestational days (Phillippi and Harrison, 2003).

Currently, the earliest pregnancy may be consistently detected using ultrasonography and palpation in gestational days 18–20. Human test kits that detect the presence of chorionic gonadotropin are of limited value due to minimal cross-reaction between the reagents and cynomolgus or rhesus chorionic gonadotropin. Radioimmunoassays for chorionic gonadotropin are available (Hobson et al., 1975); however, they are only useful for diagnosing pregnancy during gestational days 17–33, since chorionic gonadotropin secretion is limited to that time in cynomolgus or rhesus monkeys (Hein et al., 1989).

Female cynomolgus and rhesus monkeys exhibit menstrual bleeding at the end of a nonfertile cycle, when the corpus luteum has ceased producing progesterone. However, because menstrual bleeding is minimal, simple observation of the animal or the cage floor is not always sufficient to detect the presence of bleeding. Daily swabbing of the exterior vagina with cotton-tipped swabs provides an excellent means of following the menstrual cycle. Females rapidly learn to "present" for

the swabbing so that data from an entire room can be collected in a few minutes. The length of the menstrual cycles of female cynomolgus monkeys is 28.6 ± 3.3 days and normally could vary up to 20% of the previous cycle (Oneda et al., 2003). As such multiple cycles (90–180 days of monitoring) may be required to establish a definitive pattern.

Following successful mating, ultrasonography can be used to monitor the general condition of the fetus/embryo by monitoring the heart rate and developmental landmarks. Additionally, measurements such as gestational sac, greatest length, humerus and femur length, biparietal diameter, occipitofrontal diameter, head circumference, and abdominal circumference can be collected during gestation, providing an excellent method to determine stage of pregnancy (Conrad et al., 1989; Farine et al., 1988; Tarantal and Hendrickx, 1988). Accurate dating of pregnancy (within ± 2 days of conception) is possible at early stages of pregnancy. This process permits dosing at specific gestational stages. Radiography and fluoroscopy are useful for determining potential fetal abnormalities or organ dysfunctions. Both instruments, particularly ultrasound, can be utilized to perform size and functional aspects of cardiac flow, rhythm, and other cardiovascular phenomena in a fetus.

Female fertility studies (segment I) in nonhuman primates are performed by monitoring the menstrual cycle and sex hormone (estrogen, progesterone, follicle-stimulating hormone, and luteinizing hormone) profile. The pattern of expression of the sex steroid hormones in cynomolgus and rhesus monkeys is similar to humans (Hotchkiss and Knobil, 1994). Blood from female monkeys is collected every 2 days during the follicular phase and 3 days in the luteal phase, at approximately the same time of day. Samples are generally collected for one cycle prior to treatment, one cycle during treatment, and one cycle during the recovery period. Additionally, histopathology may also be performed on reproductive organs. In segment I fertility studies in males, the parameters evaluated include sperm count, motility and morphology, testicular volume, and testosterone level in the blood. Typically, samples are collected prestudy, during dosing, and during the recovery phase. The duration of treatment in the male fertility study is approximately 90 days, or one sperm cycle. Additionally, histopathology is also performed on the reproductive organs.

As expected, there are notable challenges with the use of nonhuman primates in reproductive toxicology studies, including (1) length of gestation (approximately 155–165 for cynomolgus monkeys) (Hendricx and Dukelow, 1995), (2) low conception rate (approximately 25%) at each cycle, (3) spontaneous abortions (approximately 17% for rhesus monkeys and 17.8% for cynomolgus monkeys) (Hendricx et al., 1996), and (4) a single offspring. These are, in addition to the general challenges of using nonhuman primates of small sample size (limited number of animals within a group), the limited supply of reproductively mature/appropriate animals and high interanimal variability among populations.

Maternal endpoints for segment II studies include clinical observations, food consumption, body weight measurements, hormone profile, toxicokinetic and immunogenicity evaluation of blood and milk, and ultrasonography. For teratology evaluation (generally GD100) and perinatal studies (generally GD140 and 150), fetuses are obtained via C-section, and endpoints for evaluation include body weight, teratologic evaluations (external, visceral, and skeletal examination), skeletal examination (e.g., radiography or alizarin red), organ weight, histopathology, toxicokinetics, and serum immunoglobulin G/immunoglobulin M (IgG/IgM) analysis. Additionally, the placenta is obtained during the C-section and is evaluated for structural abnormalities.

In postnatal studies (segment III), the CNS development in the nonhuman primate is more advanced at birth as compared to humans (Wood et al., 2003). Nonhuman primate infants are obtained from live birth and are generally observed for up to one year following birth. The infant endpoints include mother–infant bonding, neonatal muscle tone, and the neonatal neurobehavioral test battery (based on Brazelton Neonatal Behavioral Assessment Scale (Golub 1984; Golub 1990)), organ weight, histopathology, toxicokinetics, and serum antibody analysis (IgG and IgM).

Immunotoxicology

Drug development pipelines in pharmaceutical and biotechnology companies contain an increasing number of compounds with immunomodulation as the expected mechanism of pharmacologic activity. For some, the targeted antigens or cellular pathways are cross-reactive or have comparable activity only in humans and nonhuman primates. In addition, some drugs induce unintentional immune suppression or activation. While immunotoxicology testing in rodents has been an established process for several decades, comparable methodologies for use in nonhuman primates have lagged behind that for rodents. Appropriate tools necessary for the identification and characterization of primate immune system effects have thus become paramount to the success of some drug development programs.

Traditional nonhuman primate toxicology evaluations include basic procedures for measuring immunomodulation through physical examination and clinical observations, clinical pathology (hematology), gross observations and histopathology of immune organs, immune organ weights, and bone marrow analysis. These methods provide an overview of morphologic and gross phenotypic changes that might be associated with alterations of the immune system, but provide only general or no information about functional changes that may be present. As such, morphologic and/or functional immunotoxicology test systems, modeled after those used in rodent immunotoxicology, are now more routinely employed in nonhuman primate immunotoxicology assessments.

Morphologic Evaluation Methods

Structurally similar and difficult to discern by traditional hematology methodology, lymphocytes represent diverse population of cells with fundamentally differing functions within the immune system. However, cell surface and intracellular antigens expressed by the subpopulations of lymphocytes, e.g., B cells, T cells, and natural killer (NK) cells, can be evaluated using techniques such as flow cytometry and immunohistochemistry (IHC) (Lappin and Black, 2003). Both flow cytometry and IHC utilize antigen-specific markers, allowing for identification and quantification of unique cell populations and subpopulations. Flow cytometry is routinely employed in nonhuman primate toxicology to assess changes in peripheral blood leukocyte subsets that would not be discernible by standard hematology. Flow cytometry allows for rapid acquisition of large amounts of data from thousands of cells, providing statistically relevant information in a relatively short time interval. Additionally, flow cytometry in primates can be repeated in individual animals over the course of the study to help define the onset, persistence, and/or recovery of effects on immune cell populations. Flow cytometry is also routinely used for similar quantification of immune cells from tissues, including those from the thymus, spleen, bone marrow, and lymph nodes. However, cytometry of immune tissues requires single cells in suspension for evaluation, so the structural integrity of tissues is lost in the preparation of tissue samples.

IHC uses similar antigen identification techniques but involves microscopic evaluation similar to that done with standard histopathology. Because IHC does not require dissociation of the tissue, morphology is preserved allowing the identification of specific subpopulations and the ability to localize cell populations within the tissue and organ of interest. Flow cytometry and IHC can be used independently or in conjunction to enhance the morphologic data derived from standard toxicological study; the additional information derived from these assessments may also provide insight into mechanisms of action of some immunomodulators.

Functional Evaluation Methods

Assays for functional alterations of the immune system in nonhuman primates are also available on a limited, but expanding basis. The complexity of immune function has prompted the

development of more general assay methods looking at whole pathways rather than individual cell functions. Current nonhuman primate immune functional assays focus on alterations in the humoral (cellular mediators, such as cytokines and chemokines) and cellular (macrophage activation, phagocytosis, antigen-presentation components) immune system, with immunotoxicity programs designed to follow current regulatory recommendations.*

Cellular Immune Response

The cellular arm of the immune system is responsible for specific antigen recognition, antigen processing, antigen presentation, and ultimately antibody production (Goldsby et al., 2000). The T-lymphocyte-dependent antibody response (TDAR) assay is used to evaluate many components of the immune system including antigen-presenting cells, T lymphocytes, and B lymphocytes, which together yield an antigen-specific humoral response. The TDAR assay has been shown to be more consistent in nonhuman primates than the comparable sheep red blood cell (RBC) assay used in rodents (Lebrec et al., 2011). The humoral immune system is activated in the TDAR using novel antigens such as keyhole limpet hemocyanin (KLH) or tetanus toxoid (TT) administered by intramuscular injection. Competence of the humoral immune system in the presence of an immunomodulator is evaluated by measurement of IgM and IgG antibody responses to the novel antigen.

Adaptive Cellular Immune Response

The main component of the adaptive cellular immune system is T-lymphocyte recognition of small (peptide) antigens on cells coupled to membrane molecules encoded by the major histocompatibility complex (MHC) (Goldsby et al., 2000). General responses of the cellular immune system to antigen exposure involve cellular activation with the release of soluble mediators (cytokines and chemokines), inflammation, cytotoxic T lymphocyte (CTL)-associated cell lysis, and/or clonal expansion of T cells. Ex vivo evaluation of cell-mediated cytokine release can be done using ELISA-based assays to measure the release of proinflammatory mediators into serum (or other biological fluids) following an appropriate stimulus or intracellular cytokine staining (ICS) and analysis by flow cytometry to determine the number and phenotype of the T-lymphocyte-producing inflammatory mediators. *In vivo* testing of the cellular immune response involves the delayed-type hypersensitivity (DTH) reaction. The DTH test requires an intradermal administration of an appropriate antigen, e.g., *Candida* or *Trichophyton*, followed by gross observation of the classic "wheal-and-flare" reaction or histologic evaluation of the inflammatory reaction at the injection site, observations which may be complemented by diagnostic assessment of inflammatory cytokine kinetics or cell activation in the local draining lymph node. T-lymphocyte clonal expansion and proliferation represents another method of evaluating functionality in the cellular arm of the immune system. These proliferation assays are typically performed *in vitro* using lymphocytes isolated from peripheral blood or tissues and are quantified via increases in relative cell numbers, incorporation of fluorescently conjugated nucleotides into the nucleus during DNA synthesis, or dilution of cellular dyes after each division upon exposure of the cells to appropriate stimuli (De Boer and Perelson, 2013). Similar to morphologically based assays described earlier, cytokine release, CTL-associated lysis, inflammatory response (e.g., DTH), or cell proliferation is measured in the presence of an immunomodulator to determine the impact, if any, on cellular immune function.

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* Committee for Proprietary Medicinal Products (CPMP) "Note for Guidance on Repeated Dose Toxicity" (CPMP/ SWP/1042/99, July 2000), and U.S. FDA, Center for Drug Evaluation and Research (2002), "Guidance for Industry: Immunotoxicology Evaluation of Investigational New Drugs"

Innate Cellular Immune Response

Innate immunity represents all immediate reactions to foreign antigens or pathogens, with participation by a wide variety of cell types including neutrophils, macrophages, and NK cells (Goldsby et al., 2000). In comparison to neutrophil/macrophage response that is evaluated by function-specific tests in rodents, this evaluation most often involves histologic examination in nonhuman primates. The standard for the assessment of innate immune function has traditionally been the NK activity assay; NK cells attach to and lyse cells expressing foreign or aberrant surface proteins, e.g., virus-infected cells or tumor cells, cells displaying certain antigen–antibody complexes (antibody-dependent cell-mediated cytotoxicity [ADCC]), or cells that lack appropriate MHC molecules. For this assay, NK cells are collected from peripheral blood or spleen of animal(s) treated with an immunomodulator *in vivo* and subsequently tested *in vitro* for lytic activity against MHC-deficient cultured cells (e.g., K562 erythroleukemia cell line). Alterations in NK cell activity are the measured endpoints to determine effects of the immunomodulator on innate immune response. As with all cell-based assays in nonhuman primates, the NK cell activity assay is subject to considerable interanimal variability compared to similar testing in inbred rodent strains. In an effort to optimize this assay, a number of test and quality control refinements have been instituted, including flow cytometry and fluorescence spectrophotometer–based test systems and efforts to more efficiently normalize sample preparation and analysis among nonhuman primate-derived samples, studies, and facilities in which the assays are conducted.

Additional test modalities to evaluate the immune system of nonhuman primates are more specific and typically involve functional tests of specific components of the immune system, i.e., specific cell types or pathways. Many of these assays are used to further refine the pathogenesis of changes identified by the screening tests described earlier; most are not available or widely used outside academic or discovery research laboratories.

Primary Pharmacology

Consistent with the rationale for the use of nonhuman primates in the evaluation of toxicity, their close phylogenetic relationship to humans is directly relevant for better understanding the underlying biology of a disease and the pharmacology associated with progression or treatment. Research in rodents (primarily the mouse and rat) and select nonrodent species (e.g., rabbit, ferret, and dog) has been of immense importance; however, nonhuman primates appear to be at least equal, if not far more important, for realizing the ultimate goal to better understand conditions or diseases that occur in humans. To highlight just a few of the many efforts, nonhuman primates have been used to investigate spontaneous or naturally occurring diseases such as arthritis, diabetes, and aging; experimental models of chronic and/or debilitating diseases such as Parkinson's, Huntington's, and stroke; and induced diseases such as viral and bacterial infections.

The rhesus monkey and simian immunodeficiency virus (SIV) have been pivotal in investigations into the transmission and progression of AIDS. As AIDS research has matured over the last few decades, studies in nonhuman primate have changed from basic research to the mechanisms of viral infection and efficacy-based studies for antiviral therapy as well as the potential for vaccination as a means to control or even to prevent AIDS transmission in humans (review by Gardner and Luciw, 2008). The biologic and pharmacologic knowledge based on more common viral-based diseases such as influenza has also benefited immensely from studies in nonhuman primates. This has included investigations into the transmission of virus and the efficacy of vaccination for seasonal influenza as well as investigations into pathogenic influenza (e.g., H1N1 and other avian and porcine strains), to better understand the infamous 1918 "Spanish" influenza strain (Baskin et al., 2009; Murphy et al., 1980; Richt et al., 2012). Similarly, extensive strides for better understanding

and controlling bacterial-based infections such as pertussis (whooping cough) have also benefited from data collected in nonhuman primate models (Warfel et al., 2012).

In should be noted that many of the points for consideration outlined in the previous sections on the handling of nonhuman primates for dosing and sample collection are directly relevant in research activities for infectious disease models. Furthermore, certain viral and bacterial vectors require extensive precautions as described for BSL-3 and BSL-4 conditions. The scope of BSL-3 and BSL-4 is beyond the reach of this discussion, and investigators considering these activities are highly encouraged to seek specific training for the handling of agents and the conduct of animal studies with these agents (Biosafety in Microbiological and Biomedical Laboratories; HHS Publication No. [CDC] 21–1112).

As the human populations for many countries become older, the prevalence of the diseases of old age (e.g., Parkinson's and Alzheimer's) and the consequences of aging (e.g., stroke and cardiovascular disease) are also expected to increase. Nonhuman primates are in many ways the most relevant species for understanding the basis of human disease as well as investigations into the pharmacologic intervention of these diseases. Some occur as a natural process within nonhuman primates. A prime example is senile plaque formation, a major histopathological feature in Alzheimer's, which has been noted in the brain of aged cynomolgus monkeys (Kodama et al., 2010). A Parkinson's-like disease can be induced in cynomolgus or rhesus monkeys with exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Consistent with human disease, monkeys show limb rigidity and tremors, bradykinesia, and altered facial expressions and reactions (reviewed by Emborg, 2007). Stroke models in nonhuman primates have also been essential in the elucidation of the acute effects that occur immediately to a stroke patient and the potential for regenerative processes for damaged neuronal tissues (Cook and Tymianski, 2012). Nonhuman primates also allow for investigations into how these insults impact normal neuronal function and how treatments may improve cognition, i.e., the mental processing of memory, comprehension, and problem solving. These endpoints are at best difficult, and most often impossible, in rodents or many common research species.

The increased incidence of diabetes and other diseases associated with obesity has become a significant concern for human health. Type II diabetes and cardiovascular abnormalities (atherosclerosis and cardiomyopathy) occur as a natural disease within captive and purpose-bred nonhuman primate colonies (Wagner et al., 2006). A generalized obesity can also occur in cynomolgus, rhesus, and other nonhuman primate species (Harwood et al., 2012). However, as one would expect, even when nonhuman primates are housed under the artificial situation of captivity, obesity, diabetes, and other metabolic diseases are infrequent in nonhuman primates. As a result, the number of animals available for research and the evaluation of treatment modalities are limited. To increase the number of appropriate animals for investigation, further manipulation has been included, such as the feeding of high-fat diets that can induce hyperlipidemia with associated changes in insulin sensitivity (diabetic-like condition) and atherosclerosis (cardiovascular disease) in nonhuman primates (Jorgensen et al., 2013; Mubiru et al., 2011).

Whether a disease occurs by a natural process, exploits a natural process to accelerate the appearance and progression, or is induced by surgical, chemical, or dietary manipulation, appropriate steps must be taken to assure proper handling and housing of nonhuman primates. Diabetic animals may require insulin or specific diets to assure optimal health. For those diseases of old age (e.g., Alzheimer's), housing conditions may require modification to allow for access to food and water and socialization. Animals may become more fragile to handling, and dosing procedures may require adaptation, as well as the collection of blood or other biological samples. As with any research study, the optimal care and consideration of animal health and welfare must be a fundamental consideration and continue to be the best means of obtaining meaningful and relevant data.

PATHOLOGY

Stewart B. Jacobson

A number of texts (Benirschke, 1983; Bennett et al., 1998; Dunn, 1968; Griesemer, 1976; Jones et al., 1993; Scott, 1992; Wolfe-Coote, 2005) and journal articles (Kaup, 2002; Lowenstine, 2003) address the general subject of nonhuman primate pathology and can serve as useful resources for the reader. The intent of this section is to provide the toxicologist who has little or no knowledge of nonhuman primate pathology with enough information to acquaint him or her with the subject for those species most commonly used in toxicology. Also, the intent is to provide adequate references such that an individual can pursue additional reading as needed. Many of the references at the end of this chapter are themselves heavily referenced and will provide additional information. Additionally, a web-based resource is the Primate Info Net (WPRC, 2012) maintained by the National Primate Research Center at the University of Wisconsin.

In most instances, nonhuman primates used for toxicological studies are screened for specific disease agents before being assigned to a study. Screening is accomplished by the vendor before shipment and by the laboratory animal veterinary staff after arrival at the testing facility. Screening for these diseases provides some assurance that the animals will be in generally good health when the study is initiated. Should clinical signs of disease be observed, the veterinary clinical staff should be consulted and recommendations for treatment followed. It is important to remember that routine screening does not identify or eliminate all pathogenic and latent microorganisms. If a specific study might be compromised by particular biologic agents, the toxicologist should communicate this to the veterinarian so that proper screening can be performed and appropriate animals selected. Depending on the agents to be excluded, specific pathogen-free macaques are available only in limited numbers and can be cost prohibitive.

This section will outline only the most common diseases in four species: the rhesus (*Macaca mulatta*) and the cynomolgus (*Macaca fascicularis*) macaques, the squirrel monkey (*Saimiri sciureus*), and the common marmoset (*Callithrix jacchus*). Other species are used in research but are generally for more specialized research and not commonly used for more routine preclinical safety assessment.

Nonneoplastic Spontaneous Diseases

Background Changes

There are a number of commonly noted background histologic findings in rhesus and cynomolgus macaques. While the etiology of these changes is generally unknown, the clinical, pathological, and toxicological significance of these findings (if any) appear to be minimal. Findings include mineralization in the brain (corpora amylacea); protein inclusions in the transitional epithelium (cytokeratin) of the urinary tract; inflammatory cell infiltrates in the skin, lung, heart, liver, salivary glands, stomach, and intestines; macrophages in intestinal villi; mineralization of the adrenal corticomedullary zone, ovary, and renal papilla; multinucleated cells in the renal pelvis and liver; herniation of intestinal glands into gut-associated lymphoid tissue (GALT); reticuloendothelial hyperplasia in the spleen; lymphoid hyperplasia of the spleen and lymph nodes; involution of the thymus; and an ectopic thymus.

Bacterial Diseases

Bacterial infections are endemic in nonhuman primates and can be a significant cause of clinical disease depending on the health status of the subject population; of these the most prominent are the enteric organisms and respiratory pathogens. Nonhuman primates may be screened for

the common bacterial pathogens prior to placing them on study, procedures that are conducted by the laboratory animal veterinarian.

Shigellosis (Bacillary Dysentery)

Shigellosis is the most common bacterial disease of captive primates. The rhesus macaque is most susceptible, followed by the cynomolgus monkey. *Shigella* spp. only produce overt disease in primates, with *S. flexneri* and *S. sonnei* as the most common species isolated from nonhuman primates. The primary sources of infection for nonhuman primates are human carriers, contaminated food, diseased animals, and asymptomatic carriers. While asymptomatic carriers are common, clinical cases are most often observed when the animals are captured, transported, or otherwise experience physiologic stress. *Shigella* is readily spread from active cases to other animals in the same enclosure.

The clinical manifestations of shigellosis are variable. Carriers may be asymptomatic or may have softer and more frequent stools than normal. In more severe cases, animals sit in a hunched position, likely experiencing abdominal discomfort. Progressive dehydration, weakness, and prostration can occur quite rapidly and emaciation may become quite pronounced. In the severe form of shigellosis and dysentery, the general state of health deteriorates rapidly, resulting in anorexia and prostration. In this instance, the stools are usually liquid, containing mucus, and frequently there is evidence of frank blood. The feces in shigellosis have a distinctive odor and prolapse of the rectum is a common clinical sequelae. If left untreated, hypothermia ensues and untreated animals die in 2–3 days. In some cases, the acute form of this disease stabilizes and evolves into chronic colitis, manifested as a relapsing or chronic debilitating disease with recurrent fluid diarrhea and severe weight loss (both frequently unresponsive to general supportive care). *Shigella* is typically not isolated from the feces of animals with chronic colitis, and shigellosis is usually related to metabolic perturbations and is terminal in nature.

The pathological changes of shigellosis are restricted to the large intestine, and affected animals typically do not become septicemic. These features help differentiate *Shigella* infections from those caused by *Salmonella* and *Campylobacter* spp., the other common enteric pathogens of nonhuman primates. Shigellosis characteristically results in purulent, hemorrhagic necrotizing inflammation in the gut that appears grossly as ulcers, hemorrhage, and fibrinous pseudomembranes on the mucosa. The serosal surface may be covered with petechial hemorrhages, and the mucosa is often diffusely edematous. Changes may affect any part of the large intestine and may be focal, multifocal, or diffuse. The *Shigella* spp. infecting nonhuman primates can also produce dysentery in humans, although instances of proven transmission from monkey to humans are rare. While the clinical and pathological features of shigellosis are characteristic, etiologic diagnosis depends on culture.

Campylobacteriosis

Campylobacter species are widespread in laboratory animals and are important not only from the disease seen in animals but also from the zoonotic potential of this pathogen. *Campylobacter jejuni* has been recognized as a primary cause of diarrhea and postinfectious neurologic diseases in humans. As with other enteric pathogens, fecal–oral spread is the principal route of exposure and subsequent infection. Primates may acquire the infection in the wild, during the holding process for export, or during transport. The organism has been isolated from several species of primates, including the macaques, baboons, and marmosets. Clinically, signs vary from none (subclinical) to a severe diarrhea. The incubation period is from 2 to 5 days; persistent or intermittent watery diarrhea, without blood or mucus, is the primary symptom in macaques.

At necropsy, a mild to severe colitis may be seen, and the small intestine is sometimes affected as well. Microscopic findings are variable, but usually include infiltration of the mucosa with

mononuclear inflammatory cells and hyperplasia of the mucosa. The toxicologist must realize that stressful procedures may contribute to clinical campylobacteriosis and that measures for personal protection should be used. Etiologic diagnosis depends on culture and response to specific therapy. *Campylobacter* are frequently isolated from asymptomatic animals; as such, they can be a source of transmission to susceptible animals or a zoonotic disease.

Salmonellosis

Salmonella species, once common, are now unusual causes of dysentery in nonhuman primates. The disease may occur as a mixed infection with *Shigella* or *Campylobacter*. The serotypes involved are variable but belong chiefly to groups B, C, D, and E. In groups B and D, *Salmonella typhimurium*, *S. stanley*, and *S. enteritidis* are the most pathogenic. The usual sources of infections are contaminated food and water, usually with rodent or bird feces, healthy carriers, and sick animals. The occurrence of the disease is usually sporadic, but epidemics sometime occur.

Clinically, the disease has an acute beginning characterized by anorexia, prostration, diarrhea with mucus and blood, watery stools, and hypothermia in the more severe cases. At necropsy, the small intestine is frequently involved, in addition to the colon. This is in distinct contrast to shigellosis. Pyogranulomas may occur in the liver and other organs. Etiologic diagnosis depends on identification via bacteriologic culture.

Bacterial Pneumonia

Pneumonia is a common disease of all nonhuman primates, although the incidence in macaques has been greatly reduced with the elimination of the respiratory mite, *Pneumonyssus simicola*, through the widespread use of ivermectin. Pneumonia is often a complication of some other disease or stressful environmental condition. It may, however, occur as a primary infection, especially in young monkeys up to 6 months of age. Organisms that can be involved are *Streptococcus pneumoniae*, *Staphylococcus*, *Haemophilus influenzae*, *Bordetella bronchiseptica*, *E. coli*, *Pasteurella*, and *Klebsiella pneumoniae*.

Clinically, coughing, sneezing, and rhinitis suggest pneumonia, although individually caged animals may show few overt clinical signs. An increased body temperature is characteristic of this disease. Radiologically, the affected areas of the lungs show increased opacity and the appearance of shadows in the lung parenchyma. At necropsy, the affected portions of the lung lobes, typically the dependent or diaphragmatic lobes, are dark red or consolidated in appearance. The affected lobe(s) will typically not collapse when the thorax is penetrated and observed. Histologically, an alveolar exudate is usually prevalent and consists predominantly of neutrophils admixed with erythrocytes, desquamated epithelial cells, and fibrin. While the pathological features of many bacterial infections are characteristic, definitive etiologic diagnosis is based on bacteriologic culture.

Tuberculosis

From a colony management and zoonotic disease standpoint, the most important bacterial pathogenic organism in nonhuman primates is undoubtedly *Mycobacterium*. *M. tuberculosis* and *M. bovis* cause similar disease patterns in these species. TB is primarily a human disease, and nonhuman primates become infected by being exposed to infected humans, often in the country of origin. Old-world monkeys (rhesus, cynomolgus) are in general more susceptible to TB than new-world monkeys (squirrel, marmoset). TB can rapidly spread throughout a colony of nonhuman primates, and humans have become infected from monkeys that are subclinical in appearance.

As in humans, the primary routes of infection are usually in the respiratory or GI tract, depending on the route of exposure. The infection readily spreads throughout the body by hematogenous

and lymphogenous routes. In nonhuman primates, TB is usually pulmonary and may run a fulminating course. The disease state is characterized by numerous firm or hard, light to gray or yellow tuberculous nodules (granulomas) in the lungs, lymph nodes, liver, and spleen and may involve other organs due to dissemination of the infectious organisms. When sectioned, there is a characteristic caseous necrotic center that helps differentiate nodules (tubercles) from abscesses, which have a purulent center. In contrast to humans, tubercles in monkeys rarely calcify. Microscopically, the characteristic lesion of TB is the tuberculoid granuloma, which consists of a necrotic center surrounded by macrophages that have undergone epithelioid cell change, multinucleated giant cells, and lymphocytes. Acid-fast stains are used to detect the characteristic acid-fast bacilli, although they may be quite difficult to find. While the pathological changes are often characteristic, definitive diagnosis depends on culture or PCR identification of the infectious agent. There are other nontuberculous mycobacterial infections of nonhuman primates caused by *M. avium/intracellulare*, *M. leprae*, or other atypical *Mycobacterium* spp. These infections are pathobiologically distinct from TB and should not be diagnostically confused. A rigid testing program of all nonhuman primates and of all personnel in contact with them is necessary for adequate diagnosis and control of the TB.

A review of the mycobacterial infections of nonhuman primates has been published (Hines et al., 1995).

Viral Diseases

Viral diseases are important to the toxicologist from two points of view. First, the expression of primate viruses may interfere with a toxicity study and can, at the very least, make the data variable, questionable in nature, or difficult to interpret. Second, many of these viral organisms pose a threat to human health. Thus, the viral status of nonhuman primates must be considered as part of the animal selection and prescreening process.

Although there are approximately 75 nonhuman primate viruses that are distinctly simian (Kalter, 1983), relatively little is known about them or their impact on their hosts. Indeed, the natural host of several simian viruses is unknown, and their pathogenesis is not understood (Kalter, 1983). Two excellent discussions of the simian viruses, their hosts, and the diseases they cause are provided by Kalter et al. (1988). This section discusses only those that can be hazardous to the conduct or interpretation of preclinical toxicology studies or to the individuals handling the animals (or their tissues and bodily fluids) during the course of study conduct. In addition, a few viruses that pose a distinct threat to other primates are reviewed.

1. *Cercopithecine Herpesvirus I (Herpes Simiae, B Virus)*
   Cercopithecine herpesvirus I, otherwise known as herpes simiae, herpes B virus, or simply B virus, affects only macaques, including the rhesus and cynomolgus monkey. The organism belongs to the herpesvirus group, which is one of the largest of the viral groups. Herpes simiae is a natural infection of macaques and is endemic in most captive colonies and is the macaque homologue of herpes simplex I infection of humans. As such, it usually causes only minor lesions that resemble fever blisters in macaques and, like many herpesviruses, becomes a lifelong and typically latent infection in neural ganglia. It should be noted, however, that infected monkeys may shed virus in saliva intermittently throughout their life, with or without lesions being present. Herpes simiae is spread primarily through bites or scratches. The incidence of infection in primates is age related, being highest in adults and lowest in juveniles, with a rapid increase noted during the onset of puberty. Like many herpesviruses that infect aberrant hosts, herpesvirus simiae is highly pathogenic for humans and is the reason that the presence of this zoonotic virus is a significant health risk.

   The clinical manifestation of herpes simiae disease in macaques is variable. The natural, primary infection closely resembles herpesvirus simplex infections in humans and is usually minimal and escapes detection during the initial phases of infection. The primary infection will occasionally result in larger vesicles and ulcers on the oral mucous membranes and esophagus. Signs of a

systemic infection can develop, with interstitial pneumonia and necrosis in the liver, spleen, and other organs, typical of many systemic herpesvirus infections in other species. After the primary infection, most animals become infected for life, but seldom show any signs of infection. Macaques can shed herpes simiae virus with or without the presence of mucocutaneous or oral lesions, and most infected macaques become seropositive and remain so for life. Diagnosis of active shedding can be made by viral culture of oral mucous membranes. Postmortem diagnosis can be made by viral culture of lesions or by culture or PCR evaluation of ganglia. It is important that the toxicologist understand that all personnel in contact with conventional macaques and their blood, fluids, and tissues are potentially at risk for exposure to B virus. If humans become infected, they develop a severe and often fatal CNS infection. No vaccine is available, although postexposure antiviral therapy has been effective in a few cases.

It would be ideal to use only herpes simiae–free macaques in research, but these animals are only available in limited numbers and eradication programs are problematic in colonies where the disease is endemic. Therefore, until the supply of viral-negative animals can be greatly increased, the prevention of transmission of herpes simiae to humans is the most appropriate way to control this zoonotic disease. The toxicologist and all technical and support staff should become thoroughly familiar with their institution's biosafety protocols. Additional information on herpes B infections may be found in Cohen (2002) (Occupational Health and Safety in the Care and Use of Nonhuman Primates [2003]; Guidelines for prevention of Herpesvirus simiae (B virus) infection in monkey handlers [1987]).

2. *Simian Varicella Virus (Delta Herpesvirus, Medical Lake Macaque Virus, Liverpool Vervet Monkey Virus)*

The simian varicella viruses are a group of closely related herpesviruses that naturally infect some species of old-world monkeys, including macaques and African green monkeys, and are homologues of human varicella zoster virus, the cause of chicken pox. They are transmitted via the respiratory tract and may cause a herpetic rash, depression, and respiratory difficulty. In advanced cases that are studied at necropsy, vesicles on the skin, oral mucous membranes, and esophagus are commonly seen. Focal necrosis in the lungs, liver, spleen, lymph nodes, adrenal, bone marrow, and intestinal tract is common. Intranuclear inclusion bodies are present. Like many herpesviruses, simian varicella becomes latent in ganglia. Simian varicella virus sometimes appears in colonies because latently infected monkeys become stressed or immunosuppressed and have clinical recurrences. When this occurs, they are infectious reservoirs for uninfected cohorts.

Additional details may be found in Bladely et al. (1973), Roberts et al. (1984), and Mahalingam et al. (1992).

3. *Herpesvirus Tamarinus (Herpes T)*

Herpesvirus tamarinus, first isolated from South American marmosets, infects only new-world monkeys and is the squirrel monkey homologue of herpes simplex in humans and herpes simiae in macaques. Squirrel monkeys are the natural reservoir host, show little disease, and become infected for life. Other susceptible monkey species include *Saguinus* species, *Ateles* species, and some *Cebus* species. Transmission of the disease is by contact. Transfer of the virus to susceptible aberrant hosts, such as marmosets or owl monkeys, causes a systemic fatal disease. Fortunately, humans do not appear to be susceptible to infection with herpes tamarinus. Different species of monkeys should never be mixed or allowed to contact one another.

The lesions occurring in owl monkeys and marmosets have been described by Hunt and Melendez (1966) and are typical of systemic herpesvirus infections in many species. The clinical course in marmosets and owl monkeys is rapid, with death occurring in 4–5 days. No specific clinical signs are associated with this rapid-progressing disease, and there is no known treatment.

4. *Hepatitis*

Of the common forms of human viral hepatitis, hepatitis A and B are of most concern in nonhuman primates used in toxicology. Regarding hepatitis A virus (HAV), one must distinguish between human HAV and the closely related (but genetically distinct) endogenous viruses in cynomolgus monkeys, African green monkeys, and most likely other Asian and African species of nonhuman primates. Owl monkeys and chimpanzees are susceptible to human HAV and have been used as models for viral pathogenesis and preclinical research. Of more concern to toxicologists is the

natural HAV that commonly infects macaques, particularly cynomolgus monkeys. The spontane-ous disease in rhesus or cynomolgus is not clinically evident; the animals remain clinically normal. Transient increases in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in the serum are noted. Increases in values for these liver transaminases can produce confounding results when they occur during the conduct of a toxicity study (Lankas and Jensen, 1987; Slighter et al., 1988), leading the toxicologist to ascribe test article toxicity when there is an increased hepatic enzyme activity associated with periportal hepatic inflammation secondary to HAV. The animals seroconvert to a positive antibody status and have transitory immunoglobulin M (IgM) levels dur-ing the convalescent period. There is no obvious way of differentiating this disease-induced change from toxic effects.

The pathological lesions in the liver are minimal and described as generalized activation of sinusoidal lining cells, focal hepatocellular necrosis with occasional acidophilic bodies, and cuffs of mononuclear cells in portal areas. The hepatocellular necrosis is most often minimal, although severe cell necrosis can occasionally be found. Proliferation of bile ductules can also be found when portal inflammation is maximal. During recovery, pigment deposits can be seen in Kupffer cells and they persist for some time. Chronic hepatitis never develops. The virus is shed in the feces for an indeterminate period.

The zoonotic potential of the endogenous HAVs to macaques is unclear. While human HAV has been transferred between humans and infant chimpanzees, there is no clear evidence of transmis-sion of nonhuman primate HAV to humans or of human HAV to macaques. A vaccine for human HAV is available and some have recommended that primate handlers be vaccinated. However, it is unknown whether humans are susceptible to nonhuman primate HAV and whether the vaccine would be protective if they are susceptible.

Genetically distinct, endogenous hepatitis B viruses (HBVs) occur in humans, chimpanzees, gibbons, orangutans, gorillas, and woolly monkeys. Of these species, only chimpanzees are occa-sionally used in toxicology studies. In addition to their endogenous viruses, chimpanzees are sus-ceptible to human HBV and have been widely used as a model for hepatitis B. Because of this, most chimpanzees currently in captivity have been inoculated with human HBV and may be chronically infected; as a result, they can be a potential biohazard for HBV infection to humans. HBV infection is contracted parenterally, so the precautions in place at most primate facilities should be adequate for protection. A vaccine for HBV is available and is appropriate for those in contact with infected chimpanzees or their blood or tissues and is a common preventative vaccine for all personnel work-ing with nonhuman primates.

The primate hepatitis viruses have been reviewed in Robertson (2001).

5. *Rhesus Lymphocryptovirus, HVMF1*

In nonhuman primate, Epstein–Barr virus–related gammaherpesviruses, including rhesus lympho-cryptovirus in rhesus monkeys and HVMF1 in cynomolgus monkeys, are common natural infec-tions in many species. Most infections are latent and do not produce clinically apparent disease. In immunodeficient animals, lymphocryptoviruses have been associated with lymphomas and with squamous epithelial proliferative lesions on the skin and mucous membranes. The neoplasms are extranodal systemic B-cell lymphomas that resemble immunodeficiency-related (AIDS-associated and transplantation-associated) lymphomas in humans.

Additional information can be found in Baskin et al. (2001), Fujimoto et al. (1991), and Schmidtko et al. (2002).

6. *Callitrichid Hepatitis*

Callitrichid hepatitis occurs in common marmosets and tamarins and is caused by a rodent virus, lymphocytic choriomeningitis (LCM) virus. LCM virus is endemic in mice worldwide. Callitrichids become infected by the common practice of feeding them neonatal mice ("pinkies") or through contact with mouse urine or oral secretions. Infected monkeys may be found dead or may die after showing weakness and anorexia for several days. They may develop neurologic signs (typically described as seizures) and respiratory distress. At necropsy, jaundice, subcutaneous and intramus-cular hemorrhage, hepatosplenomegaly, and pleuropericardial effusions are seen. Microscopic changes consist of hepatocellular swelling and necrosis, lymphocytic and neutrophilic infiltrates, acidophilic bodies, and portal phlebitis. Other possible lesions include meningitis, encephalitis,

gliosis, necrosis in the spleen and lymph nodes, and interstitial pneumonia. No inclusion bodies are present. The disease is preventable by only feeding mice from sources known to be free of LCM virus (Montali et al., 1995).

SRVs

1. *SRV (D Virus, SRV Type D)*
   All type D retroviruses identified to date are of primate origin and are natural infections of animals in the wild. At least five serotypes of the SRV family infect macaques (SRV-1 through SRV-5). SRV-1 is more common in rhesus, while SRV-2 is more common in cynomolgus and pigtail macaques. Endogenous type D viruses occur in squirrel monkeys and langurs, and related proviral sequences have been identified in African and Asian colonies. The endogenous viruses appear to be nonpathogenic in the host species. The exogenous viruses infect many species of macaques, naturally occurring infection in the wild being demonstrated in *M. fascicularis* (Indonesia, but not those from the Philippines or Seychelles Islands), *M. nemestrina* (Indonesia), *M. radiata* (India), *M. tonkeana* (Sulawesi), and *M. mulatta* (China). The incidence in captive colonies varies from colony to colony, but can be quite high depending on the country of origin, source colony management procedures, and animal age. Virus can be isolated from peripheral blood mononuclear cells by coculture on Raji cells.

   Type D viruses infect B cells, T cells (CD4+ and CD8+), macrophages, epithelial cells (the salivary gland, the intestine, oral Langerhans cells), and choroid plexus. The virus is shed in saliva, and transmission requires direct physical contact or contact with fomites. Biting, licking, and grooming behaviors among cohorts are probably the usual modes of transmission, although vertical transmission can occur. Some monkeys (probably infected near birth) become persistently infected but antibody negative, serving as healthy carriers. Because of this, animals must be screened repeatedly by ELISA and PCR (or culture) to ensure they are virus-free. Experimental formalin-killed whole virus and recombinant vaccines have been used successfully. Strict husbandry procedures to prevent spread by fomites are essential to any eradication program.

   Type D viruses induce an immunosuppressive disease in macaques that may be epizootic in previously naive populations or may be enzootic. Exposed animals may develop an antibody response that clears the infection (although virus-negative, antibody-positive animals may still harbor virus in the bone marrow or gut) or become intermittently virus positive with or without antibodies. These animals may develop an acute or protracted immunodeficiency disorder with or without fibroproliferative lesions. Retroperitoneal fibromatosis and subcutaneous fibrosarcomas have been associated with SRV-2, with neutropenia, anemia, and terminal lymphopenia common sequelae. Some animals develop persistent generalized lymphadenopathy; a subpopulation of these animals eventually develop diarrhea, weight loss, bacterial infections, and/or opportunistic infections (CMV, cryptosporidia, candida, noma). B-cell lymphomas secondary to SRV infection have occurred in cynomolgus monkeys, but not in the rhesus macaque. The clinical outcome in an infected individual is directly related to the antibody response. Monkeys that die early in the course of infection have no antibody response but high levels of circulating viral antigen. Monkeys that survive with persistent viremia develop intermediate levels of antibody and have intermediate serum concentrations of viral antigen. Monkeys that clear the infection have a high level of antibody response and typically no detectable levels of viral antigen. Further, some animals apparently recover from infection. As a result, it is believed that neutralizing antibody formation is important in protection against SRV. Lesions that appear to be caused directly by type D viruses include lymphoid hyperplasia, which evolves into atrophy, nonsuppurative enteritis, sialadenitis, and myositis.

   Type D virus antigens have very rarely been detected in humans, and the biological significance of this finding is unclear.

   SRV has compromised many research studies by causing anemia, altered immune responses, altered expression of cell surface markers, impacted cytokine profiles, and a variety of nonspecific histological changes in many tissues. In the cynomolgus macaque (but not the rhesus monkey), the extensive lymphoid hyperplasia induced by SRV may progress to lymphoma. For studies that involve critical measures of immune function or that require that animals be maintained for an

extended period of time, it is essential that SRV- and antibody-negative monkeys be used. However, infected animals may have unpredictable viral counts or antibody titers at any particular point in time, and their testing status may change frequently. Therefore, many institutions require at least three negative tests, including tests for antibody and virus (culture or PCR), over a period of several months. The best solution is to use monkeys from colonies that are known to be (and are maintained as) SRV-free.

The type D retroviruses have been reviewed in Lerche et al. (2003) and Guzman et al. (1999).

2. *Simian T-cell Leukemia Virus Type I*

Simian T-cell leukemia virus type I (STLV-I) is closely related (90%–95% homologous) to the human T-cell leukemia virus type 1 (HTLV-I), the etiologic agent of adult T-cell leukemia/lymphoma, tropical spastic paraparesis, and HTLV-associated myelopathy. There is a high incidence of natural infection in many wild and captive old-world monkeys, including baboons, African green monkeys, patas monkeys, multiple species of macaques, and chimpanzees. The incidence of infection correlates with age, reaching a peak in aged animals generally over 16 years old, and is higher in females than males. Viral transmission occurs by sexual contact or parenteral inoculation. Neonatal transmission is probably unusual. Persistent infection without seroconversion has not been observed. STLV-I typically infects CD4+ T cells in macaques and CD8+ T cells in African monkeys, but some infected T-cell lines express neither marker. Although most infected animals remain latently infected and asymptomatic for life, STLV-I has been associated with lymphoma/leukemia in baboons and African green monkeys. Most investigators believe STLV-1 is not pathogenic in Asian monkeys, including macaques. There is some evidence that STLV-I may alter macaque cell surface markers and cytokine profiles, but any effects STLV-I may have on the macaque immune system have not been well defined. For routine toxicological studies in macaques, the STLV-1 status of animals is of limited biological significance. However, in studies that require detailed measurements of immune parameters, the use of STLV-1-free macaques should be considered.

3. *Simian Immunodeficiency Virus*

SIV belongs to the lentivirus subgroup of retroviruses and is a common natural cause of infections of many species of African nonhuman primates. Wild populations of Asian and South American monkeys are not infected with these viruses. SIV is of particular interest because it is the origin of the human immunodeficiency virus, the infectious agent associated with human AIDS. SIVs are well adapted to their natural hosts and do not appear to cause disease in African monkeys. However, if some isolates of SIV are inoculated into Asian macaques, they develop a disease very similar to human AIDS; as such, this experimental animal system is widely used as a model for human AIDS. SIV is unlikely to be present in captive populations of cynomolgus or rhesus monkeys unless intentionally introduced.

Additional information may be found in Apetrei et al. (2004a,b) and Gardner (2003).

4. *Other Viruses*

There are numerous other viruses that infect various species of nonhuman primates. A comprehensive listing and discussion of these is beyond the scope of this publication. Modern production facilities, quarantine procedures, and institutional practices should prevent these from adversely impacting toxicology studies in most cases. The toxicologist should consult a laboratory animal veterinarian or veterinary pathologist with knowledge and experience in primate diseases for more detailed information.

Parasitism

Parasitism is one of the most common infectious problems in nonhuman primate colonies. The incidence of parasitism in animals that are wild caught is much higher than those raised in captivity. However, even animals derived from well-managed, purpose-bred colonies are commonly infected with protozoan and metazoan parasites, although clinical disease due to parasites is relatively uncommon when compared to wild populations. Many of the background histological changes commonly encountered in monkeys used in toxicology studies are likely due to parasitism and the related immunological responses in associated tissues; these include mononuclear and

eosinophilic cell infiltrates in some tissues, lymphoid hyperplasia, and granulomas. If one considers metazoan (nematodes, cestodes, and trematodes) and protozoan parasites, along with the species they infect, the number of parasites to consider becomes too large for an adequate treatment in this chapter. Therefore, only the most common infections will be discussed. An excellent review of the pathoparasitology of nonhuman primates has been published (Toft, 1986). The chapter provides numerous references for those who wish to know of a specific parasite or group of parasites related to these species of laboratory animal.

Strongyloidiasis

Parasites of the genus *Strongyloides* are found in both old- and new-world primates. *Strongyloides cebus* has been found in squirrel monkeys and *S. fuelleborni* in the rhesus and cynomolgus macaques. Only adult female parasites and larvae are found in the intestinal tract of infected animals, while migrating larvae are found in other organs. The life cycle is complex, consisting of free-living and parasitic forms.

Diarrhea is the most common clinical manifestation of the disease, coupled with dehydration, hypoactivity and listlessness, vomiting, and emaciation. Gross lesions are characterized as a catarrhal to hemorrhagic enterocolitis. Pulmonary hemorrhage may be seen as a result of migrating larvae. Microscopically, the small intestinal mucosa contains numerous parasites and hemorrhage. This disease is diagnosed by identification of typical larvae in the stool or by demonstration of adult females, eggs, and larvae microscopically.

Oesophagostomiasis

Parasites of the genus *Oesophagostomum* are common nematodes in old-world monkeys. The adult parasites are located in the large intestine and the life cycle is direct. Adult *Oesophagostomum* cause little damage, with the primary lesions being attributed to the larval forms of the parasite. Continued reinfection results in host sensitization of the host to the parasite. Monkeys with severe infection may show a general unthriftiness and a general debilitation. However, infected monkeys are usually asymptomatic and the infection remains clinically unapparent.

Lesions at necropsy consist of smooth brown or black nodules, varying from 2 to 4 mm in diameter, which are most frequently observed on the serosal surface of the large intestine and cecum. Older nodules usually are white due to mineralization of the contents. Microscopically, the nodules contain larvae surrounded by brown debris, inflammatory cells, and a fibrous capsule. These are frequently seen, incidental findings in monkeys used for toxicology studies.

Pulmonary Nematodiasis

Metastrongylids in the genera *Filaroides* and *Filariopsis* are commonly seen in new-world primates, especially in squirrel monkeys. The infection is rare in animals raised in captivity, as the life cycle of the parasite involves the common earthworm. The adults are found in the terminal bronchioles, respiratory bronchioles, and pulmonary alveoli, and gross lesions are subtle. When present in fairly large numbers, the pleural surface of the lung has numerous, random, small, elevated, subpleural nodules. Microscopically, there is atelectasis and foci of chronic inflammatory cells.

Filariasis

Filariasis is caused by a wide variety of nematodes that are commonly encountered in new-world primates, especially squirrel monkeys. At least 12 different species have been described (Mathiesen et al., 1978) and include four species of *Dipetalonema* and seven species of *Tetrapetalonema*.

These parasites live in the abdominal or thoracic cavities or in the subcutaneous tissues of the host. They also may be found in the mesentery, along the pleural lining of the lung, and inside the pericardium. Typically, parasites in the serous cavities cause a slight peritonitis or pleuritis, with fibrinous or fibrous adhesions. In most instances, the infection is subclinical, and diagnosis is based on the demonstration and identification of the adult worms in the body cavities or subcutaneous tissues or the characteristic microfilaria in the blood.

Gastrodiscoides hominis

Gastrodiscoides hominis is a fluke that affects *Macaca spp.* and humans. Snails are the intermediate host. Macaques become infected by ingesting metacercariae encysted on vegetation. Gastrodiscoides is usually asymptomatic, although large numbers may occasionally cause mucoid diarrhea. Adults are often found in the lumen of the cecum and colon of macaques used in toxicology studies.

Pulmonary Acariasis

Lung mites, notably *P. simicola*, were once an extremely common condition of wild-caught macaques, especially rhesus. With the advent of ivermectin as a treatment for nematodiasis, lung mites have nearly disappeared from purpose-bred colonies, although older monkeys frequently have residual lesions in their lungs. The infection is usually asymptomatic. Gross lesions consist of variably sized clear, yellow, or brown air-filled cysts randomly located throughout the pulmonary parenchyma. Those located near the surface of the lungs elevate the pleura. Microscopically, the lung mite lesions (also described as "mite houses") are characterized by a dilated airway surrounded by macrophages, giant cells, and eosinophils. Macrophages laden with a golden brown pigment are always present around the lesions.

Balantidium coli

Balantidium coli has been found in old-world monkeys, new-world monkeys, and apes. Many animals have *B. coli* in the colon without showing any clinical signs. Balantidium is sometimes associated with diarrhea. Organisms are often found in the lumen of the colon of normal animals and in ulcerative lesions, mucosa, capillaries, lymphatics, and mesenteric lymph nodes of animals with colitis. It may be a primary pathogen in great apes and pigtailed macaques, but is usually associated with some other pathogen in other species.

Trypanosoma cruzi

Trypanosoma cruzi (*T. cruzi*) is an American trypanosome that is the causative agent for Chagas disease. *T. cruzi* is transmitted to nonhuman primates by triatomine insects. Infection of vector insects and sylvatic hosts occurs in Central and South America as well as in the southern and southwestern United States. *T. cruzi* infection in a nonhuman primate in captivity was first noted in a gibbon from Louisiana in 1970; since that time, infection has been documented in numerous species across the southern United States. In most cases, infection is asymptomatic but can rarely result in cardiovascular changes (lymphocytic myocarditis).

Other Diseases

Like all animal species, nonhuman primates are subject to a plethora of infectious and noninfectious diseases. These should seldom be of concern to the toxicologist working in a modern,

well-managed facility whose animal care program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and supervised by an experienced primate veterinarian and veterinary care staff.

Neoplasia

Neoplasia in captive primates is uncommon, compared to other animal species used in toxicology. Spontaneous neoplasms are rarely seen in the young monkeys, and they have found to be difficult to induce in long-term research protocols. In macaques, uterine leiomyomas (fibroids) and intestinal carcinomas occur with some frequency in older animals. There is a high incidence of B-cell lymphomas in rhesus and cynomolgus macaques that have been experimentally infected with SIV and in those severely immunosuppressed for transplantation studies. There are a few reports of B-cell lymphomas in cynomolgus monkeys infected with SRV type D. B-cell lymphomas in macaques are associated with concurrent lymphocryptovirus infection. In new-world monkeys, colonic adenocarcinomas in the cotton-topped tamarin or marmoset (*Saguinus oedipus*) are the most frequent and are associated with a chronic colitis syndrome.

The only reproducible and well-characterized tumor research model in nonhuman primates is the herpesvirus saimiri/owl monkey lymphoma system. While nonhuman primates are susceptible to many carcinogens, the induction of neoplasms requires too much time and is too unpredictable to be generally useful as a model. Therefore, nonhuman primates are rarely used in carcinogenicity studies.

Reviews of neoplasms in nonhuman primates may be found in Habis et al. (1999), Lowenstine (1986), Paramastri et al. (2002), Schoeffner et al. (2000), Schmidtko et al. (2002), and Squire et al. (1979).

DRUG METABOLISM

Christopher J. Kemper

Monkeys, especially cynomolgus (*M. fascicularis*) and rhesus (*M. mulatta*) macaques, have been widely used in biomedical research for many years, and their xenobiotic metabolism has also been extensively studied. Because they are genetically and physiologically similar to humans, rhesus monkeys particularly are the most widely used nonhuman primate in basic and applied biomedical research (Gibbs et al., 2007). Macaques are favored for research in neuroscience, reproductive physiology, and aging due to their availability and long history of use as a laboratory animal (Uno et al., 2011b). But working with them is difficult. One could, for example, sacrifice a large number of rats for the sole purpose of isolating cytochrome P450 (CYP450), but few investigators have been willing to do likewise using monkeys. In addition, monkeys represent a phylogenetic class of animals rather than an individual species. Comparing drug metabolism in rhesus and cynomolgus monkeys can be like comparing rats and guinea pigs. There are considerable differences between old-world and new-world monkeys (Litterst et al., 1976; Williams, 1971, 1974), in terms of specific activity levels, though not necessarily in terms of specific CYP450 isoenzyme activity. One has to be careful of broad generalizations based on study of any one family. This section will concentrate on rhesus and cynomolgus monkeys used in toxicological research, but will include some of what is known about the Japanese monkey (*Macaca fuscata*), African green monkey (*Cercopithecus aethiops*), and marmoset (*C. jacchus*). Where such information exists, differences between members of different phylogenetic families will be highlighted.

In a classic review, Caldwell (1981) concluded that the monkey was the best model for predicting metabolism in humans. His conclusion was based on the fact that there are fewer biochemical differences between the rhesus monkey and humans than for other species. Differences in routes of

Table 9.8   PBPK Software Tools

Berkeley Madonna	http://www.berkeleymadonna.com/	Simultaneous differential equation solver that requires some math skills
Cloe PK	http://www.cyprotex.com/insilico/	Includes PBPK modeling, *in vitro* ADME/PK and toxicology, and QSAR
MAXSIM2	http://www.maxsim2.com/	Moderately priced and very entertaining PBPK software with many preprogrammed physiological parameters
PKQuest	http://www.pkquest.com/	Free interactive PKPB program with preprogrammed human and rat data
Simcyp	http://www.simcyp.com/	Population-based modeling and simulation PKPD in virtual populations
Simulations-Plus	http://www.simulations-plus.com/	Library of widely used modules including GastroPlus (absorption modeling), ADMET Predictor (biotransformation estimates), and MedChem Studio (in silico physiochemical properties)

metabolism, however, may or may not be of practical importance. For example, Cayen et al. (1985) examined the PK of tolrestat in rats, dogs, and monkeys and concluded that both the dog and the monkey were equally good models, despite large differences in metabolite profiles, for the PK behavior (AUCs and terminal half-lives) of this drug in human subjects. The dog excreted most of the drug (82%) unchanged in the urine, whereas the monkey excreted mostly conjugated tolrestat (49%) or conjugates of other metabolites (32%). In determining which species may be the best model for humans, one should examine both the routes of metabolism and *in vivo* PK behavior of the parent chemical. The monkey may not always be the best or only model for humans.

Extrapolating study results from monkeys, whatever the species, to humans is a challenge. In the past, many anatomic and physiological variables were correlated among mammals as exponential functions of body weight, using the power formula $Y = aW^b$. A more detailed explanation may be found in the drug metabolism portion of Chapter 3. With software and hardware advances over time, physiologically based PK (PBPK) and PK and pharmacodynamic (PKPD) modeling became more and more realistic. With these models, it is easier (though still very difficult) to relate safety and efficacy results obtained from distinctly nonhuman test species to what would be expected in man. For details about PBPK processes, the reader is referred to the excellent book by Peters (2012). Table 9.8 is a list of some of the software tools that are commercially available to apply these processes.

General Considerations

Metabolism is a major clearance mechanism for three quarters of the top 200 prescribed drugs in the United States. The Phase I and Phase II enzymes, CYP450s, and glucuronosyl transferases (UGTs), respectively dominate those processes, and in humans CYP3A4 and UGT are the specific enzymes that respectively oxidize and conjugate the majority of drugs (Williams et al., 2004).

There are two processes involved in disposition that need mentioning, even though they are not metabolic processes: plasma protein binding and biliary excretion (also see Chapter 1). While they are not metabolic processes, they can play a major role in disposition of a chemical. A drug tightly bound to a plasma protein, for example, will not be as rapidly metabolized. There can be wide species differences in plasma protein binding, as reviewed by Cayen (1987). In this regard, the rhesus monkey is probably the best model for humans. In general, plasma protein binding is greatest in the

Table 9.9   Summary of Some Basic Xenobiotic Metabolizing Enzyme Systems in Monkeys

Enzyme	Concentration or Activity	Comments and References
CYP450	0.5–1.3 nmol/mg m	Amri et al. (1986), Lindstrom and Whitaker (1987), Lan et al. (1983), Challiner et al. (1981), Litterst et al. (1976), and Muller-Eberhart et al. (1983); for old-world monkeys, highest in rhesus, lowest in marmoset
Cytochrome b$_5$	0.31–0.32 nmol/mg m	Roberts et al. (1977)
NADPH/CYP450 reductase	80–220 nmol-min/mg m	Wide range due to methodological and age-related differences (Maloney et al., 1986; Leakey et al., 1983; Litterst et al., 1976)
MFO activities	3.3–9.2 nmol/min/mg m	Large differences between squirrel and rhesus monkeys (Litterst et al., 1976; Leakey et al., 1987; Iverson et al., 1982; Challiner et al., 1980; Lan et al., 1983)
Aminopyrine		
Aniline	0.30–0.80 nmol/min/mg m	
Ethoxycoumarin	0.60–0.90 nmol/min/mg m	
Ethoxyresorufin	0.03–0.08 nmol/min/mg m	
EH		Pacifici et al. (1981) (1983); species differences, highest in baboon
Styrene oxide	14–32 nmol/min/mg m	
UDP-UGT		Leakey et al. (1983), Litterst et al. (1976)
1-Naphthol	16–20 nmol/min/mg m	
4-Nitrophenol	7–25 nmol/min/mg m	
Glutathione S-transferase		High activity in rhesus monkeys (Summer and Grier, 1981)
2,4-Dinitro-1-Chlorobenzene	2.5–7 μmol/min/mg c	
DCNB, ethacrynic acid	7 to 33 nmol/min/mg c	
Protein estimates		Lan et al. (1983), Lindstrom and Whitaker (1987)
Microsomal	18–36 mg/g liver	

Abbreviations: mg m, mg microsomal protein; mg c, mg cytosolic protein.

human and lowest in the mouse. For example, clofibrate is 97% and 95% protein bound in human and rhesus monkey plasma, respectively, whereas it is only 35% bound in the mouse. Some key parameters of hepatic xenobiotic metabolism in the monkey are highlighted in Table 9.9.

Ohta et al. (1989) compared the "activation" of four representative mutagens in the Ames test, using microsomes from rats, dogs, monkeys, and humans. In general, monkey microsomes did not have the highest activity with any of the substrates, and most resembled those of the human with regard to both absolute activity and activity relative to the rat or dog.

Another consideration in studying species differences is the use of genotyping versus phenotyping (Testa and Krämer, 2010). Both are complimentary in information and need their own analytical tools. There are many reasons for species differences in drug metabolism despite the fact that most (if not all) enzymes have common ancestor genes. Differences are based on genetic variations leading to differences in enzyme expression levels, enzyme function and activity, different substrate specificities, and different catalytic rates that are present in the liver at vastly different concentrations. Genetic and environmental factors are not always distinguishable when taking the phenotype approach. The advantage of the latter is that it provides direct information on the metabolite pattern and on enzyme function and activity. This chapter, therefore, will provide both genetic and phenotypical information. For example, none of the rat 2D enzymes or homologues (the genetic information) have the same substrate preferences as human CYP2D6 (the phenotype information). The term "homologue" refers to a related gene, which can be identified on the basis of sequence similarities, whereas "orthologue" is generally reserved for a related gene that maintains functional similarities.

Phase I Reactions

Cytochrome P450: MFO Enzyme Systems

General Background

Cytochrome CYP450s (CYP450 enzymes) form the single most important metabolic enzyme system in mammals, with many families and subtypes identified (Tables 9.10 and 9.11). Although all members of this superfamily possess highly conserved regions of amino acid residues, there are relatively small differences in the primary amino acid sequences of the CYPs across species. However, even small changes in the amino acid sequences can give rise to profound differences in substrate specificity and catalytic activity (Martignoni, 2006). In most P450 subfamilies, cynomolgus, rhesus, and Japanese monkey P450s are closest to each other, followed by African green monkey P450s, reflecting the evolutionary relationship of these primate species (Uno et al., 2011b). It should be noted that, in each subfamily, P450s of these genera/species are much closer to humans than other species P450s. Additional information may be found on the CYP450 homepage (http://drnelson.uthsc.edu/CytochromeP450.html).

Concentrations of CYP450 run between 0.5 and 1.3 nmol/mg protein. Macaques, such as the rhesus, tend to have the highest concentrations, whereas marmosets tend to have the lowest. Litterst et al. (1976) reported that the amounts of CYP450 and NADPH/cytochrome C reductase and

Table 9.10   CYP450 Isozymes

CYP450 Subfamily	Human Homologue[d]	Monkey (Various Species) Homologue[b,d,s]
CYP1A[f,h]	1A1, 1A2 (5%[a])	1A1[g], 1A2
CYP1B	1B1[c]	
CYP2A[i,l]	2A6, 2A7, 2A13	2A23, 2A24
CYP2B[m,r,k]	2B6 (25%[a])	2B6, 2B17[k]
CYP2C[b,j]	2C8(1%[a]), 2C9(11%[a]), 2C18, 2C19(4%[a])	2C8, 2C9, 2C19,2C20, 2C43
CYP2D	2D6 (30%[a])	2D17, 2D19, 2D29, 2D30
CYP2E	2E1[e] (4%[a])	2E1[e,o,p]
CYP3A[l] (52%[a])[b]	3A4, 3A5, 3A7, 3A43	3A5, 3A8[q,r], 3A21[q]

Sources: Baillie, T. A. and Rettie, A. E., *Drug. Metab. Pharmacokinet*. 26(1), 15, 2011. Epub 2010 October 22; Testa, B. and Krämer, S. D., *The Biochemistry of Drug Metabolism: Conjugations, Consequences of Metabolism, Influencing Factors*, Wiley-VCH, Zurich, Switzerland, 2010.

[a] Percentage of drugs affected in human PK.
[b] Additional members of the monkey subfamily are listed in Table 9.9.
[c] Guengerich et al. (2003).
[d] Weaver et al. (1994).
[e] Chhabra et al. (1999).
[f] Edwards et al. (1994).
[g] Van Der Burght et al. (1998).
[h] Kastner and Neubert (1994).
[i] Pearce R et al. (1992).
[j] Ohmori et al. (1994).
[k] Ohmori et al. (1998).
[l] Ohmori et al. (1993a).
[m] Ohmori et al. (1993b).
[o] Amato et al. (1998).
[p] Schulz et al. (1998).
[q] Igarashi et al. (1997).
[r] Bullock et al. (1995).
[s] Ohi et al. (1989).

Table 9.11 CYP450 Genetic Profiles of Cynomolgus and Rhesus Monkeys

Gene Name	Aliases/Other Designations	Summary/Substrates (Additional References: Yan et al., 2011; Arlt et al., 2002; Uno et al., 2011a,b)
CYP1A1		Retinol, steroid metabolism, 7-ethoxycoumarin, benzo(a)pyrene, mutagenic activation of heterocyclic amines [95%, 94%]
CYP1A2		7-Ethoxycoumarin, heterocyclic amines [95%, 93%]
CYP1B1		Procarcinogen metabolism, steroid synthesis
CYP2A23		Caffeine, retinol metabolism; chemical carcinogenesis, coumarin [human CYP2A6, 95%, 92%]
CYP2A24		Caffeine, retinol metabolism; chemical carcinogenesis, coumarin [human CYP2A6, 96%, 95%]
CYP2A26		Coumarin [human CYP2A6, 94%, 93%]
CYP2B6	CYP2B17, CYP2B30	Retinol, xenobiotic metabolism, testosterone (16β-OH), bupropion [94%, 91%]
CYP2C8	CYP2C20, CYP2C74	Paclitaxel, diclofenac [95%, 92%]
CYP2C9	CYP2C43, CYP2C83	Testosterone (17-oxidation), S-mephenytoin [94%, 93%]
CYP2C18		S-mephenytoin [96%, 96%]
CYP2C19	CYP2C75	Tolbutamide, testosterone (2α-OH), pitavastatin, diclofenac [94%, 92%]
CYP2C76		It has been suggested that this CYP is a monkey-specific CYP gene partly responsible for species differences between monkeys and man; tolbutamide, testosterone (2α, 16α-OH), pitavastatin (lactone) [human CYP2C9—77%, 71%]
CYP2D17		Bufuralol, dextromethorphan [human CYP2D6—94%, 93%]
CYP2D44		Bufuralol, dextromethorphan [human CYP2D6—93%, 91%]
CYP2E1		4-Nitrophenol, chlorzoxazone, 2-hydroxylase; chemical carcinogenesis [95%, 94%]
CYP2F1	CYP2F6	
CYP2R1		Vitamin D 25-hydroxylase
CYP3A4	CYP3A8, CYP3A21, CYP3A64	Midazolam, nifedipine, testosterone (6β-OH), alprazolam, diclofenac [95%, 94%]
CYP3A5	CYP3A66	Midazolam, nifedipine, testosterone (6β-OH) [94%, 91%]
CYP3A7		
CYP3A43		Midazolam [97%, 97%]
CYP4A11		[96%, 95%] [human CYP4A22, 95%, 92%]
CYP4F2	CYP4F45	[95%, 94%]
CYP4F11		[93%, 91%]
CYP4F12		[93%, 92%]
CYP7A1		PPAR signaling pathway
CYP11B1		CYP450 11B1, mitochondrial; C21-steroid hormone biosynthesis, progesterone = > corticosterone/aldosterone
CYP17A1		Steroid 17-alpha-hydroxylase and 17,20 lyase (dehydroepiandrosterone [DHEA] is produced from pregnenolone by the successive 17-alpha-hydroxylase and 17,20 lyase activities by this enzyme)
CYP19A1	EGK_17491	Aromatase CYP450; C19/C18-steroid hormone biosynthesis, pregnenolone = > androstenedione = > estrone
CYP26A1		
CYP27A1	EGK_04803	Sterol 26-hydroxylase, mitochondrial
CYP27B1	EGK_03856	25-Hydroxyvitamin D-1-alpha-hydroxylase, mitochondrial

Sources: Uno, Y. et al., *Drug Metab. Rev.*, 43(3), 346, 2011b; PubMed Gene [http://www.ncbi.nlm.nih.gov/pubmed] and GenBank [http://www.ncbi.nlm.nih.gov/genbank/] databases, accessed on June 30, 2013.

Note: Sequence identity of P450 cDNA and amino acids between cynomolgus monkeys and humans are indicated, respectively, between the brackets [cDNA%, aa%].

microsomal mixed function oxidase (MFO) activities (with ethylmorphine, aminopyrine, aniline, biphenyl, or benzo[a]pyrene) were higher (roughly 2.5-fold) in rhesus than in squirrel monkeys. Ohmori et al. (1984) isolated and purified CYP450 from (Japanese) crab-eating monkeys (macaques). They observed that the monkey's MFO has activity against several typical MFO substrates, but antibodies against monkey CYP450 did not inhibit rat MFO activity, suggesting that there may be little structural homology between the two species. Using SDS-PAGE techniques, Challiner et al. (1981) compared microsomal preparations of marmosets treated with different inducing agents. Thus, CYP450 in monkeys exists, as in all other animal species examined, as a family of different isozymes with differing substrate specificity and sensitivity to different inducing agents. For a review on species-related differences in MFO activity, see Kato (1979). Dohi et al. (1973) reported that the CYP450 content and the nicotine-metabolizing capability were higher in rhesus monkeys than in dogs. Amri et al. (1986) compared the MFO of eight different species, including cynomolgus monkeys, to that of humans. The monkeys had essentially the same amount of CYP450 (approx. 0.75 nmol/mg microsomal protein) as the guinea pig and more than twice that of the human subjects (0.31 nmol/mg microsomal protein). When the maximal velocities were examined with six different model substrates, the monkey consistently had higher activities than the human. In fact, the similarities between the rat and the rhesus monkey were greater than those between the rhesus and the human. In general, the rhesus monkey tends to have higher amounts of CYP450 than the rat. For example, Lan et al. (1983) noted that rhesus monkeys had CYP450 concentrations comparable to that of the rat on a protein basis (approx. 1 nmol/mg), but given that the monkey had higher microsomal protein concentrations, monkeys had the higher concentrations on a gram liver basis (18.8 nmol/g).

As far as the molecular nature of the other components (e.g., NADPH/cytochrome C reductase, cytochrome b_5) of the MFO of monkeys, Schmucker and Wang (1986) isolated and purified the reductase from rhesus monkeys and found that it exists as a single protein with a molecular weight of approximately 77 kDa, which is similar to that of other species. There is immunological cross-reactivity between the NADPH/cytochrome C reductases of the pig and rhesus monkey. Using the techniques available at the time, Nobrega et al. (1969) compared the molecular properties of cytochrome b_5 from new-world monkeys (*Alouatta fusca*), pigs, chickens, and humans. The properties of the enzyme isolated from the mammalian species were quite similar with regard to molecular weight (10,600–11,400), amino acid composition, trypsin digestion pattern, and spectrophotometric spectra.

Definite gender- and age-related (post-maturation or senescent) changes in the MFO have been identified in rats. The data published by Litterst et al. (1976) did not identify any sex-related differences in rhesus MFO (either in enzyme components or activity) nor for that matter, in glutathione S-transferase, uridine diphosphate glucuronosyltransferase (UDP)-UGT, or N-acetyl transferase. Sutter et al. (1985) studied the influence of age on the MFO in female pigtail macaques (*Macaca nemstrina*) and identified no age-related (2.5–21.0 years) changes in CYP450 content, NADPH/cytochrome C reductase, or aryl hydrocarbon hydroxylase activity. Maloney and colleagues (1986) examined adult rhesus monkeys ranging from age 1 to 25 years for age differences in the MFO. They established that there were no age- or sex-related differences with regard to the amount of CYP450, MFO activity with ethylmorphine, or the phospholipid content of endoplasmic reticulum.

It would appear, therefore, that age- and sex-related changes in the MFO are not marked in monkeys. A possible age-related increase in cytochrome C reductase activity has been identified by Maloney et al. (1986) and confirmed in a follow-up paper (Schmucker and Wang, 1986). There is an age-related increase in the specific activity of this enzyme in rhesus monkeys, which is not accompanied by changes in molecular weight or immunoprecipitability. This is in contrast to the situation in the rat (see Chapter 3) where several investigators have shown that the specific activity of NADPH/cytochrome C reductase declines with age. Developmental (pre- and postnatal) changes of the MFO in monkeys have also been examined. Dvorchik and colleagues (1976, 1979) established that fetal stump-tailed macaques had higher concentrations of CYP450 and MFO activity than

those of comparably aged rats, but still had less activity than the adult animals. This observation was confirmed by Leakey and colleagues (1986). They compared the MFO of near-term fetal rhesus monkeys to both adult monkeys and adult and near-term fetal rats. The concentration of CYP450 and activity of the MFO of the fetal rhesus monkey is much higher than that of the fetal rat, both in absolute terms and relative to comparable adult levels. That is, in the monkey, the fetal CYP450 concentration was 17% of that of the adult, whereas in the rat the comparable figure was 8.5%. Differences of similar magnitude were seen with aminopyrine N-demethylase and ethoxycoumarin O-deethylase activities. Interestingly, neither fetal rats nor monkeys had measurable activity with ethoxyresorufin deethylase. This observation would suggest that this particular isozyme develops well after birth in both species.

Treatment of the monkey mothers with dexamethasone induced an increase in the MFO (enzyme components and activity toward most substrates) in fetal monkeys, but not in fetal rats. This work clearly indicates that while monkeys are born with an MFO that is better developed than that of the rat, it is considerably less active than that of the adult monkey. The monkey probably more closely resembles the human in this regard than a rodent. The inducibility by dexamethasone may provide a useful tool for studying drug-related toxicities of the newborn.

Anderson et al. (1982) reported that 4,6-diphenyl-1,10–2-[(2,4-dichloro-6-phenyl)phenoxy]ethylamine hydrobromide (DPEA) at 0.5 mM effectively inhibited the *in vitro* microsomal metabolism of N-phenyl-2-naph-tylamine in rhesus monkeys. Muller-Eberhart and colleagues (1983) reported that allylisopropylacetamide treatment (300 mg/kg/day sc for 4 days) will result in substantial decreases in CYP450 and MFO activity in rhesus monkeys. Unfortunately, the same treatment also inhibits heme oxygenase and increases 8-aminolevulinic acid synthesis and, therefore, is porphyrogenic. While interesting, such findings are of limited value to the investigator who wishes to design an experiment involving acute inhibition of the MFO.

Induction of the MFO has been examined. Challiner et al. (1980) reported that phenobarbital (20 mg/kg ip for 3 days) caused the expected increases in CYP450 and the MFO (*in vitro*) activities toward aminopyrine and ethoxycoumarin, but not ethoxyresorufin, in marmosets. *In vivo*, this induction presented as an increase in the urinary excretion of 6-O-hydroxycortisol. This latter finding suggests a noninvasive method for monitoring induction in monkeys.

This same group (Challiner et al., 1981) also examined the inducing effect of 3-methylcholanthrene (25 mg/kg ip for 3 days) in marmosets. They observed slight but significant increases in CYP450 and NADPH/cytochrome C reductase, no change in microsomal aminopyrine N-demethylase, a 2.2-fold increase in ethoxycoumarin O-deethylase, and a 10-fold increase in ethoxyresorufin O-deethylase. This is fairly typical of how other species respond to 3-methylchanthrene. Unlike phenobarbital, induction with 3-methylcholanthrene is not associated with an increase in 6-β-hydroxylation of cortisol.

Iverson et al. (1982) reported that cynomolgus monkeys respond to chronic treatment with both aroclor 1248 and 1254 (2–5 mg/kg po, three times per week for at least 10 weeks) with an approximately twofold increased in CYP450 with downward shifts in the absorption maximum. Depending on substrate, increases in activity ranged from 155% (with aminopyrine) to 630% (with ethoxyresorufin) of control. Thorgerisson et al. (1978) reported that treatment of male rhesus monkeys with 3-methylcholanthrene (a single 80 mg/kg dose 24 hours before sacrifice) caused a 57% increase in CYP450, with the expected downward shift in the maximum absorbance (450–448) and large increase (fivefold to eightfold) in benzo(a)pyrene hydroxylation activity.

Despite the fact that monkeys have been shown to respond to the classic inducing agents (phenobarbital, PCBs, etc.), there are some isolated reports in the literature that may suggest that the monkey is not as inducible as other animal species. Lindstrom and Whitaker (1987) compared the action of an aromatase inhibitor (LY 56110) in the rat, dog, and rhesus monkey. The chemical was a potent inhibitor of MFO in the rat. Two weeks of treatment results in substantial MFO induction in the rat, but not in the monkey.

CYP450 Subfamily Specifics

CYP1a Subfamily — Table 9.11 shows that cynomolgus CYP1A1 and CYP1A2 cDNAs are 95% identical to human cDNAs and have similar substrate specificities, including 7-ethoxycoumarin O-deethylase (EROD). In addition, Japanese monkey CYP1A2 cDNA, nearly identical to cynomolgus CYP1A2 cDNA, has been identified, and its protein catalyzes EROD and phenacetin O-deethylase (POND), which are mediated by CYP1As in humans (Narimatsu et al., 2005). Given that CYP1As are also found in other primates, these results suggest functional similarities of CYP1As between primates and humans.

As in humans, omeprazole induced CYP1A1 mRNA in cynomolgus monkey hepatocytes, but dexamethasone and rifampicin did not, and is regulated by AhR receptor in macaques and humans (Nishimura et al., 2007). CYP1A2 mRNA, however, is not induced by omeprazole. In general, CYP1A is also induced by β-naphthoflavone *in vivo* (Bullock et al., 1995).

CYP2 Subfamily — Similar to human CYP2A6 mRNA, cynomolgus CYP2A23, CYP2A24, and CYP2A6 mRNA are predominantly expressed in the liver, and the respective proteins substantially catalyze coumarin 7-hydroxylation (Uno et al., 2007). In other species:

1. Japanese monkey CYP2A23 cDNA was identical to cynomolgus and rhesus CYP2A23 cDNAs.
2. CYP2A mRNAs and proteins are expressed in the African green monkey liver.
3. Marmoset CYP2A-like proteins are induced by phenobarbital in the liver (Uno et al., 2011a).

While these finding are suggestive, more work needs to be done to make comparisons definitive.

Cynomolgus CYP2B6 cDNA, almost identical to human, has been identified and its mRNA is predominantly expressed in the liver. The enzyme catalyzes the same substrates (see Table 9.11). A similar rhesus CYP2B6 cDNA was isolated, and its corresponding protein substantially catalyzes testosterone 16β-hydroxylation, as in human. High sequence identities and similar tissue expression pattern and enzymatic properties suggest molecular and functional similarities of CYP2B6 between macaques and humans. Although characterization is not complete, similarities between human CYP2B with that of African green monkey brain and liver and marmoset liver enzymes have been reported (Lee et al., 2006; Schoedel et al., 2003; Schulz et al., 2001).

CYP2C enzymes are an important class of enzymes in human, metabolizing roughly 15% of drugs on the market. In the cynomolgus monkey, CYP2C8 cDNA has been identified, and the mRNA is expressed in the liver and its protein substantially catalyzes paclitaxel 6α-hydroxylation, a marker reaction of human CYP2C8 (Uno et al., 2006, 2007b). Rhesus CYP2C8 cDNA and amino acid sequence suggest similar functionality. CYP2C18 activity in macaques, however, indicates their contribution to overall drug metabolism in the liver is very limited, with little similarity to human CYP2C18 enzyme.

The major human CYP2C enzymes, 2C9 and 2C19, have cDNAs that are highly similar to cynomolgus and rhesus cDNAs. But the proteins expressed from mRNAs have different enzymatic properties, and their substrate specificities do not correspond perfectly to human CYP2Cs. Functionally, however, macaque CYP2C9 and CYP2C19 do share similar metabolic properties with human CYP2C9 and CYP2C19 (Matsunaga et al., 2002; Mitsuda et al., 2006).

Among the cynomolgus CYP2Cs identified to date, CYP2C76 mRNA is most abundantly expressed in the liver (Uno et al., 2006) and metabolizes tolbutamide and testosterone but not paclitaxel by 6α-hydroxylation or S-mephenytoin by 4′-hydroxylation, showing substrate specificity different from other cynomolgus and human CYP2Cs. A rhesus CYP2C76 cDNA, nearly identical to cynomolgus CYP2C76 cDNA, has also been identified. Using antibodies, a human CYP2C76 homologue has not been detected. These results suggest that CYP2C76 is partly responsible for the differences between macaques and humans in the metabolism of a number of drugs.

CYP2D6 is the second most important P450 in humans, after the CYP3A subfamily, metabolizing about 30% of drugs currently on the market. Cynomolgus CYP2D17 and CYP2D44 cDNAs, both highly similar to human CYP2D6 cDNA, have been identified (Mankowski et al., 1999; Uno et al., 2010) and have mRNAs expressed predominantly in the liver. As in human, their proteins metabolize bufuralol and dextromethorphan efficiently, suggesting that rhesus CYP2Ds share metabolic properties similar to human CYP2D6, with some differences. Rhesus CYP2D17 cDNA, nearly identical to cynomolgus cDNA, remains to be characterized. Japanese monkey CYP2D cDNAs (CYP2D29) have been identified as have CYP2D19 and CYP2D30 cDNAs in the marmoset.

CYP3A Subfamily — Over half of marketed drugs are metabolized by members of the CYP3A subfamily, primarily through CYP3A4, CYP3A5, and CYP3A7. Cynomolgus CYP3A4 and CYP3A5 cDNAs, highly similar to human CYP3A4 and CYP3A5 cDNAs, have been identified (Komori et al., 1992; Uno et al., 2007). Cynomolgus CYP3A4 mRNA is expressed abundantly in the liver, followed by the jejunum and adrenal gland, whereas cynomolgus CYP3A5 mRNA is expressed abundantly in the liver and jejunum, followed by the ovary, adrenal gland, and kidney (Uno et al., 2007a, 2010b, 2011).

Rhesus CYP3A4 and CYP3A5 cDNAs, nearly identical to cynomolgus CYP3A4 and CYP3A5 cDNAs, have been identified (Carr et al., 2006; Qiu et al., 2008). Although rhesus CYP3A5 remains to be characterized, it shows considerable substrate specificity and induction profiles, suggesting CYP3A4 and CYP3A5 are functionally similar in macaques and humans. African green monkey CYP3A4 and CYP3A7 cDNAs, highly similar to human CYP3A4 and CYP3A7 cDNAs, have been identified (Tang et al., 2007). However, there are some differences in enzymatic properties of African green monkey CYP3A4 from its human and macaque orthologues.

Peroxisomal Proliferation

Peroxisome proliferator–activated receptors (PPARs) are ligand-activated transcription factors that are involved in regulating glucose and lipid homeostasis, inflammation, proliferation, and differentiation. The PPARs α (NR1C1), β/δ (NR1C2), and γ (NR1C3) are members of the nuclear hormone receptor (NR) superfamily (Pyper, 2010). The foundation for the discovery and designation of the PPAR subfamily of nuclear receptors in the 1990s (Dreyer et al., 1992; Issemann and Green, 1990) is the cumulative work over the preceding 25 years with peroxisome proliferators (PPs), a group of structurally diverse chemicals that lower serum lipids and induce massive proliferation of peroxisomes in liver cells, with associated coordinated transcriptional activation of peroxisomal fatty acid β-oxidation system genes (Table 9.12).

Peroxisomal-inducing agents represent a class of chemicals that induce not only increases in MFO activity but also increases in peroxisomal number and associated enzyme activity, primarily in rodents. Hawkins et al. (1987) have reviewed species differences in responses to peroxisomal proliferators (typically antilipidemic agents related to clofibrate or phthalate-plasticizing agents such as di(2-ethylhexyl) phthalate [DEHP]) and found that monkeys are typically far less sensitive to such agents than rodents.

Table 9.12   Known Genes Associated with Peroxisome Proliferation Activity in the Rhesus Monkey (*M. mulatta*)

Gene Name	Aliases/Other Designations	Summary
PPARA		Peroxisome proliferator–activated receptor alpha
PPARD		Peroxisome proliferator–activated receptor delta
PPARG	PPARGAMMA	Peroxisome proliferator–activated receptor gamma

Source: PubMed Gene Database, [http://www.ncbi.nlm.nih.gov/pubmed] and GenBank [http://www.ncbi.nlm.nih.gov/genbank/], accessed on June 30, 2013.

For example, Rhodes et al. (1986) compared the effects of DEHP in rats and marmosets. Both species were given DEHP 2,000 mg/kg/day for 14 days. Rats responded with large significant increases in liver weight accompanied by large increases (sixfold) in peroxisomal number and associated enzyme activity (e.g., fourfold increase in palmitoyl CoA oxidation) that were not seen in marmosets. It would thus appear that in terms of microsomal and peroxisomal proliferation, monkeys are better models for the human responses than are rodents and may serve as useful animal models for a host of lipid- and glucose-based diseases (e.g., Hansen et al., 2011; Hotta et al., 1998; and Winegar et al., 2001).

For example, research has shown that obesity-associated type 2 diabetes mellitus (T2DM) that develops spontaneously under natural and "healthy" diet conditions during adulthood in several nonhuman primate species, such as the rhesus monkey (*M. mulatta*), closely parallels the disease affecting humans (Hansen et al., 2011). They tested aleglitazar, a dual PPARα/γ agonist designed to optimize glycemic and lipid benefits and minimize PPAR-related weight gain and edema in patients with T2DM. After 42 days, animals saw a significant increase in glucose clearance and a decrease in triglyceride plasma concentrations, with marked beneficial effects on HbA_{1c}, FPG, insulin sensitivity, TGs, LDL-C, HDL-C, and other lipid parameters.

Flavin-Dependent Mixed Function Oxidase

Not all the microsomal MFO activity is CYP450 dependent. There is a separate flavin-dependent mixed function oxidase (FMO) family that is different and distinct from the CYP450-dependent MFOs. In general, the identification of a tertiary amine N-oxide or sulfide S-oxide metabolite in urine provides an indication that an FMO may participate in the metabolism of the parent compound. The role of this enzyme in drug development was reviewed by Cashman (2008). There are species- and organ-related isozymes. There is currently no data to suggest that this enzyme is inducible in the strictest sense of the word (Table 9.13).

The FMOs appear to be well represented in at least some primates. For example, Ballard et al. (2007) report that their test compound, MK-0457, is metabolized in human, cynomolgus monkey, dog, and rat hepatocytes to two major metabolites, namely, the *N*-oxide and desmethyl products. The N-oxide was observed *in vivo* in monkeys as well, suggesting that the cynomolgus would be an appropriate model for that compound.

Aromatic amines may have three different routes of metabolism in the monkey: aromatic hydroxylation catalyzed by the MFO, N-hydroxylation catalyzed by the MFO or the FMO, and N-acetylation, catalyzed by N-acetyltransferase (discussed later). These pathways are not mutually exclusive. For example, Thorgerisson et al. (1978) reported that hepatic microsomes isolated from rhesus monkeys produce 3-, 5-, and 7-hydroxylated, as well as N-hydroxylated, metabolites from 2-acetylaminofluorene. Radomski and colleagues (1973) compared the metabolism of primary aromatic amines in dogs and monkeys. These chemicals cause bladder cancer in both species. Both species also

Table 9.13 FMO Genetic Profiles in the Rhesus Monkey (*M. mulatta*)

Gene Name	Aliases/Other Designations	Summary
FMO1		Dimethylaniline monooxygenase [N-oxide forming]
FMO2[a]	FMO4	Flavin-containing monooxygenase 2 (nonfunctional)
FMO3[b]		Flavin-containing monooxygenase 3
FMO4		Flavin-containing monooxygenase 4
FMO5		Flavin-containing monooxygenase 5

Source: PubMed Gene Database, [http://www.ncbi.nlm.nih.gov/pubmed] and GenBank [http://www.ncbi.nlm.nih.gov/genbank/], accessed on June 30, 2013.

[a] Yueh et al. (1997).
[b] Cashman et al. (2001).

produce N-hydroxy metabolites (e.g., N-hydroxy-2-naphthylamine from 2-naphthylamine), but excretion of acetylated N-hydroxy metabolites occurred only in monkeys. Thus, the N-hydroxy aromatic metabolites are primary bladder carcinogens, and 2-naphthylamine is carcinogenic in both species despite the differences in metabolism. Perhaps such chemicals cause fewer bladder tumors in monkeys than dogs because of the presence of a competing pathway (acetylation). The FMO has been reported to be active in N-hydroxylation in other species (Ziegler, 1988), but the involvement of this enzyme in N-oxidation in the monkey remains to be fully characterized.

The metabolism of secondary aromatic amines differs from that of primary amines in that the former are not acetylated nor N-hydroxylated by monkeys. As reported by Anderson et al. (1982), who examined the metabolism of N-phenyl-2-naphthylamine (P2NA) by seven different species, there is no evidence of N-hydroxylation in any species, including the monkey. All seven species formed the same two major metabolites *in vitro*, although the ratio of 6-hydroxy-P2NA to 4'-hydroxy-P2NA did vary. This ratio was 0.4 for rat, monkey, and mouse, whereas it was 1.0 for the dog and human. This is another example of how the monkey is not always the best model for humans. Anderson et al. (1982) also reported evidence that the CYP450-dependent MFO rather than the FMO is responsible for the metabolism of P2NA.

Epoxide Hydrolase

Epoxide hydrolases (EHs) have been detected in prokaryotes and eukaryotes ranging from plants to mammals (Fretland and Omiecinski, 2000; Hammock et al., 1997; Newman et al., 2005). In mammals, these include soluble epoxide hydrolase (sEH) and microsomal epoxide hydrolase (mEH). EH has been studied, but not extensively characterized, in monkeys. In general, monkeys have higher EH activity (against styrene oxide) than rodents and more closely resemble that of humans. Activity ranges from 14.8 ± 2.3 nmol/min/mg protein reported for rhesus (Pacifici et al., 1983) to 31.3 ± 1.7 for the baboon (Pacifici et al., 1981). Epoxides are substrates for both EH and glutathione S-transferase. While broad substrate specificity studies have not been done to confirm this point, the work of Pacifici and colleagues suggests that the preferred route of metabolism of epoxides in primates is through EH, not glutathione conjugation. In comparing primates to rodents in *in vivo* disposition of a specific xenobiotic, one should not be surprised to find a greater percentage of diol metabolites in monkeys (assuming equivalent MFO aromatic oxidation activity) (Table 9.14).

Miscellaneous Phase I Enzymes

There are, of course, a wide variety of enzyme systems that are responsible for Phase I metabolism. A short list is shown in Table 9.15.

As mentioned in the introduction to this section, all species have a wide variety of esterases (Leinweber, 1987) (Table 9.16).

Table 9.14 Profiles of Various EHs in the Rhesus Monkey (*M. mulatta*)

Gene Name	Aliases/other designations	Summary
EPHX1[a]	EGK_01603	Epoxide hydrolase 1, microsomal (xenobiotic)
EPHX2		Epoxide hydrolase 2, cytoplasmic
EPHX4	ABHD7	Epoxide hydrolase 4

Source: PubMed Gene Database, [http://www.ncbi.nlm.nih.gov/pubmed] and GenBank [http://www.ncbi.nlm.nih.gov/genbank/], accessed on June 30, 2013.

[a] Yan et al. (2011).

Table 9.15   Profiles of Various Phase I Enzyme Systems in the Monkey (*M. mulatta*)

Gene Name	Aliases/Other Designations	Summary
GAD 1 and 2	GAD65, GAD67	Decarboxylases
MAO A and B		Monoamine oxidase A and B: degrades monoamine neurotransmitters (serotonin, dopamine, and norepinephrine) and clears amine-containing drugs
PON1	EGK_13925	Paraoxonase 1, 2, and 3; broad substrate specificity; able to catalyze the hydrolysis of lipid peroxides and organophosphate pesticides

Source: PubMed Gene Database, [http://www.ncbi.nlm.nih.gov/pubmed] and GenBank [http://www.ncbi.nlm.nih.gov/genbank/], accessed on June 30, 2013.

Table 9.16   Profiles of Various Esterases in the Monkey (*M. mulatta*, from PubMed Gene Database)

Gene Name	Aliases/Other Designations	Summary
AADAC		Arylacetamide deacetylase
CEL		Carboxyl ester lipase (bile salt–stimulated lipase)
ESD		Esterase D; also S-formylglutathione hydrolase
CES 1, 2, 5A	CES7	Carboxylesterase; detoxifying proteins; hydrolysis of carboxylic acid esters into their corresponding acid and alcohol

Comparison of the coding sequence of CEL from gorilla and man reveals a 97% similarity. The other enzyme systems are less well defined in the monkey.

Like all other species, monkeys possess several alcohol dehydrogenase (ADH) enzymes. These enzymes metabolize alcohols to aldehydes, which can be highly reactive. The ADHs are cytoplasmic, dimeric, Zn-containing enzymes (Testa and Krämer, 2010). They are widely distributed in the body, but their highest activities are found in barriers of entry, such as the stomach and liver, and in organs that need special protection such as the brain. The preferred substrates are primary alcohols (such as ethanol) but are also able to oxidize secondary alcohols and even some aldehydes and to reduce aldehydes and ketones (Table 9.17).

Fortunately, the monkey (as well as humans) contains an abundance of aldehyde dehydrogenases and oxidases, as shown in Table 9.18, which metabolize a wide variety of aliphatic and aromatic aldehydes to various end products, generally a carboxylic acid. They are widely distributed in the body and occur as cytosolic, microsomal, or mitochondrial enzymes. Aldehydes are toxic, and interruption of their oxidation either by an alcohol overdose or by coadministration of an aldehyde dehydrogenase inhibitor such as disulfiram can be fatal. The disulfiram drug Antabuse (Hardman et al., 1995) is used as a treatment for alcoholism.

Table 9.17   Profiles of Various Alcohol Dehydrogenases in the Monkey (*M. mulatta*)

Gene Name	Aliases/Other Designations	Summary
ADH6		Alcohol dehydrogenase 6 (class V)
ADH7		Alcohol dehydrogenase 7 (class IV)
DHRS2		Dehydrogenase/reductase SDR family member 2
HSD17B10	HADH2	Hydroxysteroid (17-beta) dehydrogenase 10
HSD17B1		
ALAD		Aminolevulinate dehydratase
HSD17B3		Hydroxysteroid (17-beta) dehydrogenase 3

Source: PubMed Gene Database, [http://www.ncbi.nlm.nih.gov/pubmed] and GenBank [http://www.ncbi.nlm.nih.gov/genbank/], accessed on June 30, 2013.

Table 9.18 Profiles of Various Aldehyde Dehydrogenases in the Monkey (*M. mulatta*)

Gene Name	Summary
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1
ALDH1A2	Aldehyde dehydrogenase 1 family, member A2
ALDH1A3	Aldehyde dehydrogenase 1 family, member A3
ALDH1B1	Aldehyde dehydrogenase 1 family, member B1
ALDH2	Aldehyde dehydrogenase, mitochondrial like
ALDH3A1	Aldehyde dehydrogenase 3 family, member A1
ALDH3A2	Aldehyde dehydrogenase 3 family, member A2
ALDH3B1	Aldehyde dehydrogenase 3 family, member B1
ALDH3B2	Aldehyde dehydrogenase 3 family, member B2
ALDH5A1	Aldehyde dehydrogenase 5 family, member A1
ALDH6A1	Aldehyde dehydrogenase 6 family, member A1
ALDH7A1	Aldehyde dehydrogenase 7 family, member A1

Source: PubMed Gene Database, [http://www.ncbi.nlm.nih.gov/pubmed] and GenBank [http://www.ncbi.nlm.nih.gov/genbank/], accessed on June 30, 2013.

Phase II Reactions: Conjugation

Glucuronidation

Conjugation reactions or biosynthetic pathways are those that take the products of MFO, FMO, or EH and add an additional functionality to form a new chemical moiety. For example, the oxidation of benzene to phenol is Phase I, whereas the formation of phenyl glucuronide is a Phase II reaction. Of course, if the molecule intrinsically has the appropriate electrophilic acceptor group present, such as the terminal carboxylic acid in benoxaprofen, it can undergo Phase II metabolism directly. In the case of benoxaprofen, it is metabolized to its 1-O acyl glucuronide (Obach, 2009). As reviewed by Caldwell (1981), there are some conjugative reactions that have been shown to occur only in humans or monkeys. These include the N1-glucuronidation of methoxysulfonamides, the glucuronidation of cyproheptadine to yield a quaternary glucuronide, and the conjugation of arylacetic acids with glutamine (see the following section on amino acid conjugation reactions).

Glucuronidation is a clearance mechanism for approximately 1 in 10 of the top 200 prescribed drugs (Williams et al., 2004). In general, glucuronides are usually inactive end products, but this is not always the case. 1-O-acylglucurorides are particularly unstable and have a number of products (Janssen et al., 1982). They can be hydrolyzed to the aglycone nonenzymatically in base. In the GI tract, intestinal or microfloral glucuronidases can hydrolyze these glucuronides to aglycone, which is then available for reabsorption. This can cause a secondary absorption phase that may be observable in the concentration time profile of the molecule and can be a source for increased exposure to the original drug. This process is the primary cause of what is known as enterohepatic recirculation. If the alpha carboxylic acid is substituted, the isomer will rearrange. The glucuronic acid group can also rearrange to the 2, 3, or 4 glucuronic acid esters in both base (even at pH 7.4) and UV. These isomers are generally much more stable than the 1-O acyl glucuronide. Smith et al. (1986) have demonstrated that glucuronidation of zomepirac results in a reactive chemical moiety that binds to macromolecules, creating a potential antigen that may solicit an allergic reaction. An excellent summary on this issue was written by Wainhaus (2005). A more detailed discussion of glucuronide chemistry can be found in the UGT drug metabolism section of Chapter 3, The Rat.

Dulik and Fenselau (1987) have examined some of the aforementioned primate-specific UDP-UGT, or UGT reactions *in vitro* using immobilized enzyme preparations. Interestingly, while they confirmed *in vitro* that primates have a much higher activity in the glucuronidation of

sulfadimethoxine, the human was still found to have roughly three times the activity of the rhesus monkey. Additionally, the activity toward both p-nitrophenol and cyproheptadine was more similar between the monkey and rabbit than between the monkey and human.

Others have studied UDP-UGT activity in the rhesus monkey. Litterst et al. (1974) compared *in vitro* microsomal UDP-UGT activity in the rhesus monkey to that of the squirrel monkey. With 4-nitrophenol as the substrate, activity in both species were comparable (approximately 6–14 nmol/min/mg), whereas the squirrel monkey had considerably higher activity with 2-aminophenol (approximately 0.5 vs. 2.9 nmol/min/mg). When compared to the rat, the rhesus monkey had higher activity with p-nitrophenol and comparable activity with o-aminophenol.

Pacifici et al. (1986) established that the rhesus monkey has a relatively high transferase activity toward morphine. Leakey et al. (1983) established that the monkey has activity against a wide variety of substrates, including 4-nitrophenol and 1-naphthol. Activities in adult females were compared to those of the late-term fetus. With natural (type 2) substrates (e.g., steroid hormones on bilirubin), enzyme activities of the fetus were less than 5% than those of the adult, whereas with synthetic substrates (e.g., 4-nitrophenol), activities ran 40%–120% of those of the adult.

These results clearly suggest that UDP-UGT exists as a family of isozymes with differential development in the rhesus monkey. Also, unlike the rat, intrauterine exposure of rhesus monkeys to dexamethasone (10 mg/kg sc given to the pregnant animals 1, 2, and 3 days prior to near-term cesarean section) greatly enhanced the fetal hepatic UDP-UGT activity toward the type 2 substrates. For example, activity against bilirubin was increased from 3 to 74 nmol/mg/min. Hence, despite the evidence that UDP-UGT exists as a family of isozymes in both rodents and primates, the genetic and developmental controls are different. As mentioned elsewhere, there is stereospecificity in the activity of primate UDP-UGT. For example, when the activities were examined using either (R) or (S) lorazepam as a substrate, the rabbit had no stereospecificity, the monkey exclusively glucuronidated the (R) isomer, and human preparation preferred the (R) to the (S) isomer by approximately 4:1 (Dulik and Fenselau, 1987). Pacifici et al. (1986) also demonstrated that the UDP-UGT present in the gut plays a major role in the metabolism of morphine in the rhesus monkey (Table 9.19).

Unlike CYP3A activities, the interpretation of data from comparisons between intestinal and hepatic UGT activities is difficult due to differences in the expression profiles of the major UGTs between both tissues. In the intestine, the primary isoforms are UGT1A10 for humans and UGT1A8 for monkeys, while UGT2B7 is the primary liver isoform. Intestinal glucuronidation, however, probably coupled with efflux transporters, could play a significant role in PK. Komura and Iwaki (2011) discuss intestinal versus liver metabolism of CYP3A and UGT substrates in depth. Nishimura et al. (2008) studied the induction of human and cynomolgus mRNA of various UGTs using rifampicin, dexamethasone, and omeprazole. Their findings indicated that the pattern of mRNA expression of UGTs in cynomolgus monkey hepatocytes is similar to that in human hepatocytes and that the sensitivity to the inducers in the elevation of mRNA levels of UGTs is higher in cynomolgus monkeys.

Glutathione and Glutathione S-transferase

The important conjugative enzyme, glutathione S-transferase, has received some, but hardly exhaustive, scrutiny in the monkey. Litterst et al. (1974) compared glutathione activity S-transferase with 1,2-dichloro-4-nitrobenzene (DCNB) in rhesus and squirrel monkeys. They found that activity ranged from approximately 15 to 25 nmol/min/mg cytosolic protein with no species-related differences. Asaoka and colleagues (1977a,b) characterized glutathione S-transferase of the Japanese crab-eating monkey and the rhesus monkey. Using various chromatographic techniques, they tentatively identified five isozymes, with various activities against six typical substrates. Thus, as in other species, glutathione exists as a family of isozymes in other monkeys. The pattern present in rat appears to more closely resemble that of the human as opposed to that of the monkey (Asaoka et al., 1977a). Unlike the situation in rats where there are definite isozymic substrate specificities,

Table 9.19   Genetic Profiles of Various UDP-UGTs in the Monkey (*M. mulatta*, Unless Otherwise Indicated)

Gene Name	Summary
UGT1A1	UGT 1 family, polypeptide A1; 7-hydroxy-4-trifluoromethylcoumarin, estradiol at 3-hydroxy position, and 7-ethyl-10-hydroxycamptothecin glucuronidation are similar to human *in vitro* (Hanioka et al., 2010). Rhesus UGT1A1 was greater than 99% and 95% identical to cynomolgus UGT1A1 and human UGT1A1, respectively (Dean et al., 2002) (~15% of human UGT activity).
UGT1A4	~15% of human UGT activity; implicated in human tamoxifen metabolism but not monkey (Kaku et al., 2004).
UGT1A6	Human and cynomolgus monkey mRNA induced by omeprazole.
UGT1A8	Currently not fully characterized.
UGT1A9	Currently not fully characterized.
UGT2A1	UGT 2 family, polypeptide A1, complex locus.
UGT2A3	UGT 2 family, polypeptide A3.
UGT2B4	UGT 2 family, polypeptide B4.
UGT2B7	UGT 2 family, polypeptide B7; primary human UGT for drug metabolism (~30% of human UGT activity) (e.g., denopamine [Kaji and Kume, 2005]); propranolol glucuronidation in human distinct from cynomolgus.
UGT2B9	2 alleles isolated, 7-hydroxy-4-(trifluoromethyl)coumarin, estrogens, opioid substrates (Dean et al., 2004).
UGT2B15	UGT 2 family, polypeptide B15.
UGT2B18	*M. fascicularis* cDNA, mRNA characterized; principally active on C19 steroids; active in the liver, prostate, kidney, testis, adrenal, bile duct, bladder, colon, small intestine, cerebellum and pancreas (Beaulieu et al., 1998).
UGT2B20	UGT 2 family, polypeptide B20; androgen glucuronidation; in cynomolgus' liver, prostate, kidney, epididymis, and adrenals; 92% homologous to human UGT2B15, with similar substrate activities and tissue localization (Barbier et al., 1999).
UGT2B30	Steroid metabolism; found in the prostate, testis, mammary, kidney, adrenals, and intestine (Girard et al., 2002).

Source: PubMed Gene Database, [http://www.ncbi.nlm.nih.gov/pubmed] and GenBank [http://www.ncbi.nlm.nih.gov/genbank/], accessed on June 30, 2013.

one glutathione S-transferase (GSH-T) isozyme in the monkey tended to have the highest activity with all substrates examined (Asoaka et al., 1977a).

This enzyme was further examined by Asoaka et al. (1979). It has a molecular weight of 48,000, is composed of two identical subunits (which is consistent with the isozymic structure of glutamine sulfhydryl transferase (GSH-T) of other animals), and is competitively inhibited by hexachlorobenzene. Summer and Greim (1981) compared the activity GSH-T from the livers of the rat, rhesus monkey, chimpanzee, and human. The model substrates were 1-chloro2,4-dinitrobenzene (CDNB) and DCNB. With CDNB, rhesus had the highest activity (about 6.5 μmol/min/mg cytosolic protein) followed by the chimpanzee (3.2), the rat (1.8), and then human (1.6). With DCNB, the rat had the highest activity (about 51 nmol/min/mg), followed by the rhesus monkey (2.9), chimpanzee (9.2), and then human (4.6). This pattern suggests that (1) compared to the rat, primates have comparable or greater activity of glutathione S-transferase, depending on substrate, and (2) rhesus monkeys tend to have greater activity than humans (Table 9.20).

Acetylation

Primary amines can be acetylated (and then hydroxylated) by monkeys, but the available evidence suggests wide differences in activity with different substrates. Litterst et al. (1976) reported that *in vitro* cytosolic N-acetyl transferase activity of rhesus monkeys was about 4.9 nmol/min/mg protein with p-aminobenzoic acid, but only 0.01 nmol/min/mg with sulfadiazine. Old-world monkeys tend to have higher rates of N-acetyltransferase activity than new-world monkeys (Table 9.21).

Table 9.20   Genetic Profiles of Various Glutathione S-Transferases in the Monkey (*M. mulatta*, Unless Otherwise Indicated)

Gene Name	Aliases/Other Designations	Summary
MGST1	EGK_03433	Microsomal glutathione S-transferase 1
MGST2	EGK_16108	Microsomal glutathione S-transferase 2
MGST3		Microsomal glutathione S-transferase 3
GSTA3		Glutathione S-transferase alpha 3
GSTA4		Glutathione S-transferase alpha 4
GSTK1		Glutathione S-transferase kappa 1
GSTM1		Glutathione S-transferase mu 1
GSTM2		Glutathione S-transferase mu 2 (muscle)
GSTM3		Glutathione S-transferase mu 3 (brain)
GSTM4		Glutathione S-transferase mu 4
GSTO1		Glutathione S-transferase omega 1
GSTO2	EGK_20046	Glutathione S-transferase omega 2
GSTP1		Glutathione S-transferase pi 1
GSTT1		Glutathione S-transferase theta 1
GSTT2	EGK_21203	Glutathione S-transferase theta 2

Source: PubMed Gene Database, [http://www.ncbi.nlm.nih.gov/pubmed] and GenBank [http://www.ncbi.nlm.nih.gov/genbank/], accessed on June 30, 2013.

Table 9.21   Profiles of Various N-Acetyltransferase Enzymes in Monkey (*M. mulatta*, Unless Otherwise Indicated)

Gene Name	Aliases/Other Designations	Summary
NAT1	NAT, EGK_18730	N-acetyltransferase 1, enzyme that acetylates both arylamines and arylalkylamines (Yan et al., 2011)
NAT2	NAT, AAC2	Enzyme that acetylates only aryl amines and may have a role in carcinogenesis

Source: PubMed Gene Database, [http://www.ncbi.nlm.nih.gov/pubmed] and GenBank [http://www.ncbi.nlm.nih.gov/genbank/], accessed on June 30, 2013.

Amino Acid Conjugation

Williams (1974) extensively reviewed the conjugation of arylacetic acids with glutamine. With phenylacetic acid, for example, humans will excrete almost 95% as the glutamate conjugate. Similar results were obtained with monkeys, where, depending on species, up to 90% is excreted as the glutamate conjugate. Interestingly, this is one metabolic reaction where there are not large differences between old- and new-world monkeys. Nonprimates such as the rat will produce 80%–100% phenylacetate glycine conjugate and no detectable glutamate conjugates (Table 9.22).

Table 9.22   Profiles of Various Amino Acid Conjugation Systems in the Monkey (*M. mulatta*)

Gene Name	Aliases/Other Designations	Summary
AGXT		Alanine-glyoxylate aminotransferase
BAAT		Bile acid CoA: amino acid N-acyltransferase (glycine N-choloyltransferase)
CCBL1		Cysteine conjugate-beta lyase, cytoplasmic
GLYAT	EGK_04961	Glycine-N-acyltransferase

Source: PubMed Gene Database, [http://www.ncbi.nlm.nih.gov/pubmed] and GenBank [http://www.ncbi.nlm.nih.gov/genbank/], accessed on June 30, 2013.

Sulfate Conjugates

Sulfate ester formation (e.g., phenol sulfate formation from phenol) is catalyzed by the cytosolic enzyme 3′-phosphoadenosine-5′-phosphosulfate (PAPS)-sulfotransferase using PAPS as the cosubstrate. The reaction has important toxicological involvements. A more in-depth discussion of this metabolic path can be found in the respective section in Chapter 3 (Table 9.23).

Sulfotransferase enzymes catalyze the sulfate conjugation of many hormones, neurotransmitters, drugs, and xenobiotic compounds. These cytosolic enzymes are different in their tissue distributions and substrate specificities. The gene structure (number and length of exons) is similar among family members. The SULT1A1 gene encodes one of two phenol sulfotransferases with thermostable enzyme activity. Multiple alternatively spliced variants that encode two isoforms have been identified as well. The gene and protein sequence in rhesus monkeys both have a homology >95% to that of human. The gene and protein sequence for the other reviewed sulfotransferase, SULT2A1, has a similar homology. The SULT1A1 gene is conserved in human, rhesus monkey, chimpanzee, zebra fish, and mosquito. SULT2A1 is conserved in human, rhesus monkey, chimpanzee, cow, mouse, and rat. Results from a study by Nishimura et al. (2008) showed that primary cultures of hepatocytes isolated from the cynomolgus monkey liver are as useful as human hepatocytes for evaluating the induction of SULT1A1 and SULT2A1 in preclinical studies.

Transporters

In recent years, it has become increasingly clear that drug transporters play an important role in the absorption, distribution, and elimination (ADE) of drugs (Chu et al., 2013). These enzyme systems, much like the P450 enzyme systems, are also prone to induction and inhibition, which can be a mechanism for PK-based drug–drug interactions (DDIs). The current draft FDA Guidance on DDIs (FDA, 2012) and a similar guidance by the EMA (EMA, 2012) go into great detail about their respective agencies' understanding of the importance of these formally little regarded enzyme systems. Table 9.23 outlines the transporters found in three cell types: enterocytes, hepatocytes, and renal tubule cells. To date, the most identified transporters belong to one of two superfamilies: ATP-binding cassette (ABC) and solute carrier (SLC). Systems identified in the monkey are listed in Tables 9.24 and 9.25. In contrast to metabolizing enzymes, which are largely concentrated in the liver and intestine, transporters are present with varying abundance in all tissues in the body.

Table 9.23   Profiles of Various Sulfotransferase Enzymes in the Monkey (*M. mulatta*)

Gene Name	Aliases/Other Designations	Summary
SULT1A1		Sulfotransferase family, cytosolic, 1A, phenol preferring, member 1
SULT1A2		Sulfotransferase family, cytosolic, 1A, phenol preferring, member 2
SULT1A3		Sulfotransferase family, cytosolic, 1A, phenol preferring, member 3
SULT1B1		Sulfotransferase family, cytosolic, 1B, member 1
SULT1C3		Sulfotransferase family, cytosolic, 1C, member 3
SULT1C4	SULT1C1; SULT1C2	Sulfotransferase 1C2-like
SULT1C4	SULT1C2	Sulfotransferase family, cytosolic, 1C, member 4
SULT1E1		Sulfotransferase family 1E, estrogen preferring, member 1
SULT2A1	EGK_10823	Sulfotransferase family, cytosolic, 2A, DHEA preferring, member 1
SULT6B1		Sulfotransferase family, cytosolic, 6B, member 1
CHST7		Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7
TST	EGK_03004	Thiosulfate sulfurtransferase (rhodanese)

Source: PubMed Gene Database, [http://www.ncbi.nlm.nih.gov/pubmed] and GenBank [http://www.ncbi.nlm.nih.gov/genbank/], accessed on June 30, 2013.

Table 9.24 Genetic Profiles of ABC Transporter Systems in the Monkey (*M. mulatta* Unless Otherwise Indicated, from PubMed Gene Database) cyno = cynomolgus (*M. fascicularis*)

Gene Name	Aliases	Summary
ABCA1		Subfamily A (ABC1), member 1
ABCA2		Subfamily A (ABC1), member 2
ABCA3		Subfamily A (ABC1), member 3
ABCA4		Retinal-specific ABC transporter like
ABCB1	MDR1, P-gp	Cyno mRNA liver expression < human, intestine > human; brain microvessels 4.7 fmol/μg protein
ABCB4		MRP 3 like
ABCB6		Subfamily B (MDR/TAP), member 6
ABCB11		Subfamily B (MDR/TAP), member 11
ABCC2	MRP2	Subfamily C (CFTR/MRP), member 2; mRNA expressed in GI > human; protein expressed in liver at 1.2 fmol/μg protein comparable to human
ABCC3	MRP3	Subfamily C (CFTR/MRP), member 3
ABCC4	MRP4	Subfamily C (CFTR/MRP), member 4; mRNA enriched in the kidney compared to other organs; protein expression 1.5 fold > human
ABCC5		Subfamily C (CFTR/MRP), member 5
ABCC10		Subfamily C (CFTR/MRP), member 10
ABCC12		Subfamily C (CFTR/MRP), member 12
ABCD1		Subfamily D (ALD), member 1
ABCD3		Subfamily D member 3 like
ABCD4		Subfamily D (ALD), member 4
ABCF1		Subfamily F (GCN20), member 1
ABCG2	ABCG3, BCRP	Subfamily G (white), member 2; Cyno brain microvessels 14.2 fmol/μg protein; liver 0.20 fmol/μg protein
ABCG8		Subfamily G (white), member 8
TAP1		transporter 1, ABC, subfamily B (MDR/TAP)
TAP2		Antigen peptide transporter 2 like

Sources: Giacomini, K. M. et al., *Nat. Rev. Drug Discov.* 9(3), 215, March 2010; Bleasby, K. et al., *Xenobiotica*, 36(10–11), 963, October–November 2006; Ito, K. et al., *J. Pharm. Sci.*, 100(9), 3939, 2011; Uchida, Y. et al., *J. Neurochem.* 117(2), 333, 2011.

Transporters can also work in concert with metabolizing enzymes and play a role in drug metabolism. Genome and protein data for these enzymes can be found in PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and UniProtKB (http://www.uniprot.org).

Several allometry-based approaches (see Chapter 3) are commonly used to predict human PK, based on studies conducted in preclinical species such as rat, dog and monkey. However, these methods generally perform poorly for transporter substrates due to species differences in substrate specificity for transporters, differences in expression levels of transporters in various organs across species, and the lack of orthologues for some transporters across species. A better comprehension of these differences is therefore important to first understand mechanistically why species differences may be found in drug disposition *in vivo* and, second, to devise strategies to build translational PKPD models that can take these differences into account (Chu et al., 2013). Examples include quantitative structure–activity relationship (QSAR) models (Gombar et al., 2004) and homology modeling (Ekins et al., 2007; Ha et al., 2007).

P-glycoprotein (P-gp, ABCB1, MDR1) mediates the ATP-dependent export of drugs across the plasma membrane of cells. P-gp is expressed in various blood–tissue barriers including the luminal membrane of the small intestine, the blood–brain barrier (BBB), the canalicular membrane

Table 9.25   Genetic Profiles of SLC Family Transporter Systems in the Monkey (*M. mulatta* Unless Otherwise Indicated, from PubMed Gene database)

Gene Name	Aliases	Summary
SLC2A1		Family 2 (facilitated glucose transporter), member 1
SLC2A2		Family 2 (facilitated glucose transporter), member 2
SLC2A3		Family 2 (facilitated glucose transporter), member 3
SLC3A1		Family 3 (cystine, dibasic, and neutral amino acid transporters, activator of cystine, dibasic and neutral amino acid transport), member 1
SLC3A2		Family 3 (activators of dibasic and neutral amino acid transport), member 2
SLC5A1		Family 5 (sodium/glucose cotransporter), member 1
SLC7A11		Family 7 (anionic amino acid transporter light chain, xc- system), member 11
SLC7A4		Family 7 (orphan transporter), member 4
SLC7A7		Family 7 (amino acid transporter light chain, y+L system), member 7
SLC7A8	EGK_18020	Family 7 (amino acid transporter light chain, L system), member 8
SLC7A9	EGK_10427	Family 7 (glycoprotein-associated amino acid transporter light chain), member 9
SLC10A1	EGK_18329	Family 10 (sodium/bile acid cotransporter family), member 1
SLC10A2		Family 10 (sodium/bile acid cotransporter family), member 2
SLC15A2		Family 15 (H+/peptide transporter), member 2
SLC15A2	EGK_11349	Family 15 (H+/peptide transporter), member 2
SLC16A1	EGK_01106	Family 16, member 1 (monocarboxylic acid transporter 1)
SLC16A2		Family 16, member 2 (thyroid hormone transporter)
SLC16A3	EGK_09124	Family 16, member 3 (monocarboxylic acid transporter 4)
SLC19A2		Family 19 (thiamine transporter), member 2
SLC19A3		Family 19, member 3, thiamine transporter 2
SLC22A1	OCT1	Family 22 (organic cation transporter), mRNA expressed in kidney and GI
SLC22A2	OCT2	Family 22 (organic cation transporter), mRNA expressed in the kidney
SLC22A6	OAT1	Family 22 (organic anion transporter), member 6; kidney mRNA expression on par with human
SLC22A8	EGK_06087, OAT3	Family 22 (organic anion transporter), member 8; kidney mRNA expression on par with human; brain microvessel protein expression on par with human
SLC25A13		Family 25 (aspartate/glutamate carrier), member 13
SLC28A1		Family 28 (sodium-coupled nucleoside transporter), member 1
SLC28A2		Family 28 (sodium-coupled nucleoside transporter), member 2
SLC28A3		Family 28 (sodium-coupled nucleoside transporter), member 3
SLC29A1	EGK_14955	Family 29 (nucleoside transporters), member 1
SLC31A1		Family 31 (copper transporters), member 1
SLC38A2	EGK_03554	Family 38, member 2
SLCO1B1	OATP1B1	Solute carrier organic anion transporter family, member 1B1
SLCO1B3	OATP1B3	Solute carrier organic anion transporter family, member 1B3
SLCO2A1		Solute carrier organic anion transporter family, member 2A1
SLCO2B1		Solute carrier organic anion transporter family, member 2B1
SLCO3A1		Solute carrier organic anion transporter family, member 3A1
SLCO4A1		Solute carrier organic anion transporter family, member 4A1

of hepatocytes, and the apical membrane of kidney proximal tubule epithelia. P-gp has broad substrate specificity (Chu et al., 2013). Most of its substrates are neutral and amphipathic positively charged compounds. Like humans, monkeys have one MDR1 gene; rodents have two paralogous genes. In humans, MDR1 mRNA is predominantly expressed in the adrenal gland and kidney, with significant expression in the liver. In monkey, it is less enriched in the liver and kidney but more enriched in the GI tract. P-gp expression in human brain microvessels is similar to that in

cynomolgus monkeys, suggesting that the monkey may be a better model to predict the role of P-gp in limiting brain penetration of drugs in humans. Supporting this contention has been the observation that there is a good correlation found between human and monkey MDR1-expressing cells. P-gp-based DDIs are a concern in the clinic, especially with respect to intestinal absorption and distribution across the BBB. Current clinical data indicate that there are no consistent examples in which inhibition of P-pg in the BBB resulted in adverse effects. However, attempts by several manufacturers in the early 2000s to improve bioavailability of such drugs as paclitaxel by coadministering P-gp inhibitors such as antifungals and macrolides resulted in unprecedented CNS toxicities (unpublished data).

Breast cancer resistance protein (BCRP, ABCG2) is localized in the apical plasma membrane of many tissues in humans, including the intestine, liver, kidney, testis, placenta, and BBB (Maliepaard et al., 2001). BCRP was identified originally as a determinant of multidrug resistance in cancer cell lines *in vitro* (Doyle, 1998). It has a role in limiting oral bioavailability and transport across the BBB, blood–testis barrier, and maternal–fetal barrier of some selected substrates (Giacomini et al., 2010). Enzyme protein in monkey liver tissue approximately equals that of human (0.14 fmol/μg protein). BCRP protein expression in brain microvessels in monkey and human were 1.85× and 3.52×, respectively, higher than in mice.

Multidrug Resistance Proteins

The multidrug resistance protein (MRP) that is most commonly associated with the transport of endogenous compounds and drugs is MRP2. Its role is most clearly defined in the liver, kidney, and intestine, where it is located in the apical membrane and is involved in the excretion of many Phase II metabolites. Protein levels in the liver were 10-fold higher in rat than in other species, with rat ≫ monkey > human ~ dog (Li et al., 2009). Notable in the Li paper (and more recent publications) was the use of LCMS technology instead of less specific immunologic assays to identify these proteins. Improvements in specificity and sensitivity should result in more accurate PBPK models and better predictions.

Variation of MRP3 between human donors ranged sixfold however. MRP3 is located in the basolateral membrane in several tissues, including the enterocytes, liver, and kidney distal tubules (Keppler, 2011). In humans, the level of mRNA and protein of MRP3 in liver varies ~ 80-fold. MRP4 localization is organ specific: in human, it is detected in the apical membrane in the kidney proximal tubular cells but in the basolateral membrane in hepatocytes. While MRP4 genes have been detected in the monkey, they have not been functionally characterized.

The organic anion–transporting polypeptides (OATPs) form a large family of solute carrier organic anion transporter (SLCO) proteins within the SLC superfamily. In human liver, OATP family members expressed are OATP1B1, OATP1B3, and OATP2B1. All three are localized in the sinusoidal membrane of hepatocytes, where they are responsible for the uptake of a wide range of endogenous compounds and drugs from the portal venous blood into hepatocytes (König, 2011). Orthologues for the human OATPs are expressed in monkey, but the proteins have not been functionally characterized. The significant species differences for the OATP transporters make it challenging to translate PK or PD data from preclinical species to humans.

The organic anion transporters (OATs) belong to the SLC22 family that covers both the organic anion and cation transporters. The primary OATs are OAT1 and OAT3 and are expressed in the kidney where they play an important role in the uptake of numerous drugs. Expression of OAT1 in human and monkey is limited to the basolateral membrane of renal proximal tubular cells. OAT3, however, is located in the basolateral membrane of the proximal tubule in human. Interestingly, mRNA expression of both OAT1 and OAT3 is enriched in the kidneys of rodents, monkey, and humans, but not in dog (Bleasby et al., 2006). OAT4 is located in the apical membrane of the proximal tubule cells in humans and has been detected in monkey.

There are many members of the organic cation transporter (OCT) family. The primary members are OCT1 and OCT2, which mediate facilitated diffusion of many endogenous and exogenous cationic compounds and play a pivotal role in hepatic, renal, and intestinal excretion of drugs (Ciarimboli, 2008). OCT1 mRNA is strongly expressed in the liver of mouse, rat, monkey, and human and is located to the basolateral membrane of hepatocytes. Expression of OCT1 was not observed in the kidney of human and monkey (Bleasby et al., 2006), and there appears to be little expression in those species intestines. In human, OCT2 is located in the basolateral membrane of the renal proximal tubules (Motohashi, 2002). Monkey kidney shows similar expression of OCT2 (Ciarimboli, 2008). Given that monkey exhibits similar tissue distribution of OCTs as humans, monkey could be a better choice than rodents when selecting an animal model for predicting renally based DDIs for OCT substrates.

Given the easy availability of rodents for scientific research, initial emphasis has been on human and rodent transporters. But the amount of information for transporters from other species is increasing. Monkey (not surprisingly), in particular, shows promise as an exceptional model. With the acquisition of basic data, development of whole-body PBPK models to predict plasma and tissue tissues exposures can progress. This area, however, is still in its infancy for transporter substrates.

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The Minipig

Shayne Cox Gad and Alain Stricker-Krongrad

CONTENTS

TOXICOLOGY

Shayne Cox Gad

The use of pigs (*Sus scrofa*) in biomedical research is now well established. In toxicology, whereas the use of pigs in the United States was historically limited to dermal studies, since the mid-1990s, they have become very popular for pharmaceutical studies in place of dogs and primates. They have been extensively used for surgical (Swindle et al., 1988) and physiological (primarily cardiovascular, renal, and digestive) research (Khan, 1984; Clausing et al., 1986; Gad, 2013) for years. The preference in these uses is the minipig.

Until relatively recently, their use in toxicity testing was uncommon except in the testing of veterinary or herd-management drugs intended for use in swine or in dermal toxicity and absorption studies. This reluctance to use the pig as a model has been based primarily on body mass, which leads to the need for significantly more test compound. Because of their well-accepted physiological (and newly identified metabolites) similarities to humans, minipigs are becoming increasingly attractive toxicological models (Table 10.1). In fact, they were already more frequently used in nutritional toxicology studies (Clausing et al., 1986; Gad, 2013). Among the more common experimental animals, pigs are the only one whose use is on the increase (Khan, 1984). Their expense (both in procurement and in maintenance) and their relatively large size have mitigated against their use in more general toxicity testing. The development of minipigs has resulted in a strain of more manageable size. In addition, the increase in expense in the use of dogs, as well as the perceived lay opposition to their uses, makes minipigs even more attractive as a nonrodent species for general toxicity studies. The dog is a far more common companion animal, and many of the recent developments in animal care and use laws have made specific provisions about the care of dogs. Minipigs have been shown to be more sensitive to a wide variety of drugs and chemicals (e.g., carbaryl, methylmercury) than dogs (Khan, 1984). The Food and Drug Administration (FDA) has kept its own breeding colony of minipigs since the early 1960s. In short, there are scientific, economic, and sociological reasons that make minipigs good toxicological models. The reader is referred to an excellent short review by Phillips and Tumbleson (1986) and a recent text by McAnulty et al. (2012) that put the issue of minipigs in biomedical research into the context of modeling in general. Table 10.2 presents the advantages of the minipig.

Table 10.1   Minipigs in Toxicity Testing

Due to the many advantages, mini- and micropigs are real
  alternatives to the use of nonrodents (dogs, ferrets,
  and primates).

Minnesota minipig introduced in 1949.

Body weights at the age of 2 years

  Yucatan minipig, 70–90 kg

  Yucatan micropig, 40–45 kg

  Göttingen micropig, 35–40 kg

Used in general toxicity testing and reproduction, teratological,
  and behavioral toxicity (aspects of public acceptance as
  species for testing).

Table 10.2   Main Advantages of the Minipig

Similar to humans in

  Cardiovascular anatomy and physiology

    Ventricular performance

    Electrophysiology

    Coronary artery distribution

  Human skin

    Thickness and permeability

    Pigmentation

    Allergic reaction

    Reaction to burning and distress

  GI system, digestion, and absorption of xenobiotics

  Renal system

  Immune system (FDA: "… better than rodents")

  P450 total enzyme activity (especially CYP2E1, CYP3A4)

Several breeds of miniature swine have been developed. These include, in the United States, the Yucatan micro- and minipigs, the Handford, the Sinclair, the Pitman-Moore, and the Hormel. The Yucatan and the Sinclair tend to be the most commonly used. Panepinto and Phillips (1986) have discussed the characteristics, advantages, and disadvantages of the Yucatan minipig in some detail. In Europe, the Göttingen minipig is extensively used, but use in the United States is limited by availability. At sexual maturity (4–6 months), the typical minipig weighs 20–40 kg, as compared to 102 kg for the more common pig, 8–15 kg for the dog. Micropigs weigh about 14–20 kg at sexual maturity. The minipig and the dog have comparable life spans; for example, Peggins et al. (1984) reported that the average life span for miniature swine is 15–17 years. The average beagle dog may have a life span of 8–12 years. Most of this discussion will focus on the purpose-bred minipigs, primarily the Yucatan and the Sinclair.

Husbandry

Housing

General reviews of handling and husbandry have been described by Panepinto (1986), Swindle et al. (1988), and McAnulty et al. (2012). Young weanling pigs can be kept for short periods of time (up to 1 month) in standard dog cages with the floor modified with narrow mesh to account for the smaller foot of the pig. After that, however, their rapid growth generally makes such caging inappropriate. Larger stainless steel cages would be extremely expensive. Standard dog runs could have enough floor space to be converted for pigs, but smooth flooring does not provide appropriate footing for pigs and needs to be covered with wood chip bedding (Swindle et al., 1988). Although pigs are very social, they do not have to be group housed, as discussed by Barnett and Hensworth (1986); individually housed swine show little evidence of a chronic stress response. Insufficient space, on the other hand, can cause chronic stress in pigs. Hunsaker et al. (1984) have described an inexpensive caging system for miniature swine that is appropriate for toxicology studies. The flooring and walls are constructed of 0.50 cm welded wire coated with polyvinyl chloride polymer. As described, the unit has sufficient room for two pigs, separated by a partition. These units are relatively inexpensive and provide more than sufficient floor space (about 17 ft 2 per pig) to meet the recommendations for pigs.

Water and Feed

Like all animals, pigs should be permitted free access to potable water, preferably from a municipal water supply intended for human consumption. Drinking water intended for pigs does not have to be filtered or deionized. Various diets have been described. Because of their size (i.e., high maintenance charges and test article demands), pigs have seldom been used for chronic studies where the possibility of waterborne environmental contaminants could influence a study.

For miniature swine, the consistent use of a certified chow from a major manufacturer is recommended (Swindle et al., 1988). Free access to feed is not recommended as pigs will eat to excess. Available feed should be restricted to approximately 4% of body weight per day to prevent the animals from becoming obese.

Restraint and Dosing

In general, minipigs are docile and easily socialized and trained. Barnett and Hensworth (1986) recommended a socialization regimen of 2 min of gentle interaction (e.g., stroking, etc.). Pigs, like most experimental animals, are rarely simply kept and fed, but have to be occasionally restrained so samples can be taken and other measurements made. Restraint methods designed for commercial

swine should not be used for laboratory swine. Panepinto et al. (1983) have described a sling method that provides restraint with minimal stress. The most frequently mentioned dosing routes in the literature are dietary admix, dermal (topical), gavage, and intravenous (IV) injections. Generally, minipigs are restrained in a sling while being dosed by the active route such as gavage. If the experiment requires the implantation of, for example, an indwelling catheter, minipigs can be anesthetized with ketamine (20 mg/kg IM) as described by Swindle et al. (1988).

Clinical Laboratory

Clinical chemical and hematological parameters for minipigs have been studied. Ranges for some of the more commonly examined parameters from Yucatan minipigs are summarized in Tables 10.3 and 10.4 (from Radin et al., 1986). Parson and Wells (1986) have published similar data on the Yucatan minipig. Brechbuler et al. (1984) and Oldigs (1986) have published on the Göttingen minipig. Middleton and coworkers have published extensive lists (organized by age and sex) on the hematological parameters (Burks et al., 1977) and serum electrolytes (Hutcheson et al., 1979) for the Sinclair minipig. In general, the clinical laboratory picture of the various strains is quite similar. No real differences between sexes have been identified, but age can be very much a factor. For example, serum creatinine can be 33% higher in a 3-month-old as compared to 18-month-old Sinclair minipigs (based on data reported by Burks et al., 1977). As with other species, health status, feed composition, feeding regimen, fasting state, season, time of day, etc., can affect clinical laboratory results in the minipig. Toxicological experiments should not be run without concurrent controls.

General Toxicity Testing

The appropriateness of minipigs as a species for the general toxicity assessment of new drugs, chemicals, and medical devices has now been well established. See McAnulty et al. (2012) and Gad (2013), as well as Van Ryzin and Trapold (1980) who published on the toxicity of proquazone

Table 10.3   Minipig Clinical Chemistry Parameters in Different Strains

Parameter	Yucatan	Göttingen
Glucose (mmol/L)	3.75 ± 0.64	5.98 ± 1.01
Urea (mmol/L)	7.84 ± 2.64	3.19 ± 1.15
Creatinine (mmol/L)	155 ± 16	52.2 ± 11.1
Total protein (g/L)	74 ± 9	54.0 ± 4.6
Albumin (g/L)	50 ± 6	26.2 ± 6.0
Bilirubin total (μmol/L)	3.42 ± 1.37	—
Triglycerides (mg/L)	267 ± 134	565 ± 250
Total cholesterol (mmol/L)	1.85 ± 0.38	1.62 ± 0.38
γ-Glutamyl transpeptidase (U/L)	61.6 ± 11.2	—
Alanine aminotransferase (U/L)	72.5 ± 13.6	—
Aspartate aminotransferase	40.3 ± 5.9	—
Na^+ (mmol/L)	140.5 ± 4.2	142.3 ± 3.00
K^+ (mmol/L)	4.1 ± 0.3	3.94 ± 0.32
Cl^- (mmol/L)	103.1 ± 4.3	101.3 ± 3.6
Ca^{++} (mmol/L)	2.62 ± 0.18	2.58 ± 0.16
$PO_4^=$ (mmol/L)	2.41 ± 0.26	1.61 ± 0.30

Sources: Parsons, A. and Wells, R., *Lab. Anim. Sci.*, 36, 428, 1986; Brechbuler et al., *J. Clin. Chem. Clin. Biochem.*, 22, 301, 1984; Oldigs, B., *Swine Biomed. Res.*, 2, 809, 1986.
Note: Data are mean $\pm$ SD.

Table 10.4   Minipig Hematological Parameters in Different Strains

Parameter	Yucatan	Göttingen
Red blood cell (10^6/mm³)	7.61 ± 0.15	7.0 ± 0.80
Hemoglobin (g/dL)	14.87 ± 0.18	14.9 ± 1.2
Hematocrit (%)	44 ± 0.5	44.6 ± 4.1
Mean corpuscular volume (fL)	58.5 ± 0.8	64.4 ± 3.7
Mean corpuscular volume (pg)	19.8 ± 0.3	21.4 ± 1.3
Mean corpuscular hemoglobin concentration (g/dL)	33.9 ± 0.3	33.2 ± 0.8
White blood cell (10^3/mm³)	12.73 ± 0.41	12.6 ± 3.0
Lymphocytes (10^3/mm³)	7.25 ± 0.24	5.75 ± 1.52
Neutrophils (per mm³)	4.47 ± 0.24	5.27 ± 1.29
Eosinophils (per mm³)	534 ± 57	517 ± 31
Monocytes (per mm³)	422 ± 35	945 ± 71
Basophils (per mm³)	89 ± 15	63 ± 1.3
Platelets (10^3/mm³)	—	441 ± 119

Sources: Burks, M. et al., *Growth*, 41, 51, 1977 (12-month-old, sexes pooled); Radin, M. et al., *Lab. Anim. Sci.*, 36, 425, 1986.

(a nonsteroidal anti-inflammatory drug [NSAID]) in rats, dogs, and minipigs. Rats in general are exquisitely sensitive to NSAIDs, and proquazone was no exception; dosages of 25 mg/kg/day (13 weeks) and above caused evidence of gastrointestinal (GI) toxicity. In dogs, dosages as high as 75 mg/kg were without effect, and higher dosages cause emesis, anorexia, and anemia, but no GI lesions. In a longer-term study, however, evidence of gastric damage was produced in the dog. In minipigs, dosages ranged from 6 to 94 mg/kg/day (26 weeks). Dosage-related mortality, diarrhea, and gastric ulceration were observed at all levels. In this particular example, if the minipig had been used in place of the dog, somewhat different conclusions regarding the safety of proquazone would have been reached. Generalizing from this single case, minipigs may be more similar to rats than to human beings in their response to NSAIDs.

The toxicity of relatively large numbers of chemicals has been investigated in regular swine (summarized in Table 10.5). These studies did not use minipigs, but still may be used to infer the toxic syndrome in minipigs. After all, if pigs cannot with reasonable certainty predict the toxicity in a different breed of pig, how reliable can they be in predicting the toxicity of chemicals in human beings? In general, these publications suggest that the toxic syndromes produced in pigs reliably predict the toxicity of drugs and chemicals in human beings. There are some notable quantitative and qualitative differences. As mentioned, pigs appear to be more sensitive to the GI effects of NSAIDs (a quantitative difference with humans). Pigs primarily develop methemoglobinemia (a qualitative difference with humans) in response to acetaminophen rather than liver damage (Artwhol et al., 1988). There are now also a large body of available literature on the use of the mini swine (Gad, 2013).

Reproductive Toxicity and Teratogenicity

Whereas the rat and the rabbit will probably remain the mainstay of reproductive developmental toxicity testing, the minipig has several attractive features that may make it an appropriate model when one of these other species is not. The estrus cycle is approximately 20 days, which certainly makes the minipig a more convenient nonrodent model than dogs for reproductive toxicity studies. The gestation period is about 114 days and the critical period for organogenesis is days 11 through 35 (Hayama and Kokue, 1985). Average litter size is about six, with weaning in about 5 weeks. The piglets are born quite well developed and make very good models for behavioral teratogenicity testing. These aspects would make the pig more attractive than the monkey for teratogenicity testing.

Table 10.5   Summary of Literature on Toxicity in the Pig

Chemical (References)	Syndrome	Comments
3-Nitro-4-hydroxyphenylarsenic (Rice et al., 1985)	Subchronic dosing leads to exercise-inducible muscle tremors and colonic convulsions.	Controlled lab study, dietary admix, Landrace pigs, 20 kg
Lead (Lassen and Buck, 1979)	Only mild clinical signs despite blood levels of 240 μg/mL and decreases in ALAD, hemoglobin, and HCT.	Controlled lab study, oral administration in solution, crossbred 6-week-old pigs, 17–24 kg
Polybrominated biphenyls (Howard, Werner, and Sleight, 1980)	200 ppm in diet for 12 weeks led to decreases in lymphocyte mitogen response in sow and piglets (4 weeks postpartum). No changes in bactericidal activity. Large increases in pre-β-lipoprotein.	Controlled lab study, dietary admix, two-generational study of immunotoxicological effects
Zearalenone (James and Smith, 1982)	10 μg/g feed for 4 weeks caused increases in uterine weight with no effect on growth or feed efficiency.	Controlled lab study, dietary admix, Yorkshire gilts, included comparisons with rats
Chlorpyrifos (Scheidt et al., 1987)	Exposure of newborn piglets prior to healing of umbilical and tail wounds led to severe signs of organophosphate insecticide toxicity: lethargy, ataxia, salivation, and diarrhea.	Controlled lab study to follow up a case report, gravid, crossbred sows, aerosol exposure
Acetaminophen (Artwhol et al., 1988)	Acutely, major clinical sign of toxicity was due to methemoglobin formation. Relatively mild effects on liver—primarily dose-related increases in glycogen (500–2000 mg/kg IV over 90 min).	Controlled lab study, IV infusion, crossbred male and female swine
Gossypol (Hascheck et al., 1989)	Severe cardiotoxicity: diffuse myofiber atrophy with perinuclear vacuolation. Liver damage (marked centrilobular congestion and necrosis).	Clinical report, feedlot swine problem traced to cottonseed supplementation of feed
Fenbendazole (Hayes, Oehme, and Leipold, 1983)	When given at 200 mg/kg for 14 days, caused transient leukopenia and increased serum sorbitol dehydrogenase, but not histopathological lesions.	Controlled lab study, oral gavage, female Yorkshire pigs, 18–24 kg
Aflatoxin B_1 (Osuna and Edds, 1982)	0.2 mg/kg/day for 10 days causes increased serum alkaline phosphatase, sorbitol dehydrogenase, prothrombin time, and partial thromboplastin time. Decreases in total protein and β- and γ-globulins.	Controlled lab study, emphasis primarily on clinical pathology parameters
Turmeric oleoresin (Bille et al., 1985)	60, 296, and 1551 mg/kg/day produced dose related increases in thyroid and liver weights. Pericholangitis, hypoplasia of the thyroid, changes in epithelial cells in the kidney and bladder.	Controlled lab study, dietary admix, crossbred swine
Diacetoxyscirpenol (Weaver et al., 1981)	0, 2, 4, 8, and 16 ppm. Decreases in feed consumption and weight gain at all dosages. Multifocal proliferative, gingival, and lingual lesions. Glandular and mucosal small intestine hyperplasia.	Controlled lab study, dietary admix, crossbred weanling pigs
Toxaphene (DiPietro and Haliburton, 1979)	Ataxia, lethargy, depression, diarrhea, seizures, increased rectal temperatures.	Clinical case report, findings attributed to improper use of a topical preparation
T-2 toxin (Lorenzana et al., 1985)	Cyanosis, anorexia, lethargy, pneumonia, necrotic lymph tissue, necrotizing gastroenteritis, and other lesions. Depressed lymphocytes and macrophage function, but no effect on RBCs.	Controlled lab study

Other aspects of the porcine reproductive system make pigs good models for other types of research as well. Sows have the epitheliochorial type of placenta, which blocks the transplacental passage of proteins, and therefore, the newborn piglet is free of antibodies. The minipig is, therefore, also an excellent model for gnotobiotic research, as discussed by Mandel and Travnicek (1987). For example, Kim et al. (1980) used the gnotobiotic minipig model in their studies of natural killer cells and antibody-dependent cellular toxicity.

Numerous examples of teratogenic studies with minipigs can be found in the literature. Ivankovic (1979) studied the teratogenic effects of N-alkylnitrosoureas using the Göttingen minipig. He demonstrated, for example, that a single dose of (70 mg/kg IP) ethylnitrosourea given 13 days postcoitus to the sow causes severe bone malformations in the piglets. This chemical class is also teratogenic to rats and hamsters.

Hayama and Kokue (1985) have published on the natural incidences of various malformations and functional deficits in the Göttingen minipig; many other practical aspects of teratogenicity testing in minipigs are discussed in their review article, which is a highly recommended reading. They studied pyrimethamine (a folic acid antagonist, given in the feed at an average of 3.6 mg/kg/day on days 11 through 35 of gestation) and established that it causes a high incidence of major malformations such as cleft palate, clubfoot, and micrognathia.

Dexter et al. (1983) have examined Sinclair minipigs, which will voluntarily consume (i.e., do not avoid) alcohol, as a potential model for the human fetal alcohol syndrome (FAS). They reported that alcohol (20% in drinking water) causes a progressive decrease in litter size and piglet weight, but does not result in the more common FAS-related malformation such as microcephaly and narrowed palpebral fissures.

Dermal Toxicity

Although rabbits are commonly used for the assessment of primary dermal irritation, pigs are firmly considered the first choice model for the more sophisticated study of dermal permeability and toxicity. As reviewed by Sambuco (1985), human and porcine skin are similar with regard to sparsity of the pelage, thickness and general morphology, epidermal cell turnover time, and size, orientation, and distribution of vessels in the skin. The particularly thin haircoat and lack of pigments of the Yucatan minipig makes it particularly ideal for dermal studies. The size of the animal also provides the additional practical advantage of abundant surface area for multiple site testing.

Sambuco (1985) has described the sunburn response of the Yucatan minipig to ultraviolet (UV) light, suggesting that this species would also make a good model in phototoxicity as well as photocontact dermatitis studies. Thirty 12 cm sites were demarcated, permitting the study of 15 different dermal dosages of UV radiation.

Mannisto and coworkers (1984) have published a series of articles on the dermal toxicity of the andiralins in the minipig. In one experiment, 24 sites per minipig were used to assess the acute dermal irritation of various concentrations to four different chemicals per site. The range of concentrations tested permitted them to calculate the median erythema concentration and median irritation concentrations with relatively few animals. They were able to show clear differences between anthralin congeners (antipsoriatic drugs) with regard to irritation. When compared to other species (mouse and guinea pig), the response of the minipig was the most similar to humans in that in both species, these chemicals are delayed irritants, and several days postexposure may pass before the maximal irritant response is presented.

In a second experiment (Hanhijarvi et al., 1985), the chronic, cumulative dermal effects of anthralin chemicals were studied in minipigs. Using only 12 animals, they were able, by having 32 sites per animal, to study the effects of two different chemicals (dithranol and butantrone, both anthralins) in three different formulations at three different concentrations each. The protocol also

included observations for systemic toxicity, clinical laboratory measurements, plasma drug analyses, and gross and histopathological examinations.

In a third report (very similar to the second), Hanhijarvi et al. (1985) clearly demonstrated that the type of vehicle can greatly influence irritation in that dithranol was clearly more irritating when applied in paraffin than when applied in a gel. They were also able to demonstrate that although dithranol was less irritating than butantrone acutely, the cumulative irritations (mean scores at the end of 6 months of six times per week applications) were quite similar (Mannisto et al., 1986). There was no evidence of systemic toxicity nor of test article in plasma with either species.

Cardiovascular Toxicity

In general, the published literature consistently maintains that the cardiovascular systems of swine and humans are very similar. For example, as reviewed by Lee (1986), swine, including minipigs, have a noticeable background incidence of atherosclerotic lesions, and swine fed high-lipid diets will develop even more extensive atherosclerotic lesions. High-lipid diets will produce lesions similar to advanced atheromatous lesions seen in humans. Although few drugs or chemicals have been shown to cause atherosclerosis, this information has three general applications to toxicology and pharmacology. First, the feeding regimen of minipigs should be carefully controlled in general toxicity studies to minimize the incidence of arterial disease, especially in long-term studies. Second, the pathologist should be aware of the natural background of this disease when preparing a diagnosis. Third, the minipig could provide a convenient model for the study of atherosclerotic disease and the screening of potential therapies. These factors and the size of the animals and its cardiovascular system have made the minipig the animal of choice for the evaluation of cardiovascular devices such as stents.

The minipig has been used to study cardiotoxicity. Van Vleet et al. (1984) reported that minipigs were the only other species studied other than dogs to develop cardiac damage in response to large doses of minoxidil. In both the pig and the dog, minoxidil cardiotoxicity is characterized by vascular damage (with hemorrhage in the arterial epicardium) and myocardial necrosis (mostly of the left ventricular papillary muscles). Interestingly, in the dog the atrial lesion is largely restricted to the right atrium, whereas in the pig, it is restricted to the left atrium. These lesions can be produced in roughly 50% of the minipigs given 10 mg/kg of minoxidil for 2 days and sacrificed 48 hours after the last dose (Herman et al., 1986, 1988). Herman and colleagues have published extensive descriptions of minoxidil-induced lesions in minipigs in comparison to those produced in dogs (Herman et al., 1988, 1989). The right versus left arterial difference is believed to be due to differences in the anatomical pattern of coronary circulation between two species (Herman et al., 1988).

Minipigs are also sensitive to the cardiotoxic effect of doxorubicin. When given six IV injections of either 1.6 or 2.4 mg/kg of doxorubicin at 3-week intervals, minipigs develop cardiac lesions similar to those seen in dogs, rabbits, and other experimental animals (Herman et al., 1986). The lesion is characterized by cytoplasmic vacuolation and varying degrees of myofibrillar degeneration and loss. Thus, the minipig is sensitive to the cardiotoxic effect of two well-known and extensively studied chemicals. Therefore, it is a suitable nonrodent species for the general assessment of the cardiotoxicity.

Advantages and Disadvantages

There are two disadvantages to the use of minipigs. The first is their size. Although minipigs are smaller than regular swine, at maturity they are generally larger than beagle dogs. The second is their expense: they are not only larger than dogs but currently carry higher purchasing and maintenance costs. Among the advantages are the facts that they are long-lived, cooperative animals with

well-defined physiological and metabolic characteristics. As they are not either popular companion animals (like dogs) or physically resemble humans (like monkeys), minipigs are not specifically discussed in animal "welfare" laws like the other two species. Depending on their final form, new animal welfare regulations could make the space and maintenance costs for dogs and monkeys very prohibitive. This may make minipigs increasingly more attractive as a nonrodent species for general toxicity testing.

PATHOLOGY

Alain Stricker-Krongrad

Pathology of the Göttingen Minipig: Background and Incidentally Occurring Changes—Introduction

Pathological evaluation of toxicity studies is concerned not only with the recognition of lesions caused by treatment directly but also with the identification of spontaneous lesions that may have increased in severity and/or frequency in treated animals. It is, therefore, vital to be aware of the background and incidentally occurring lesions that can be observed during macroscopical and microscopical examinations of organs from laboratory animals in routine toxicity studies.

Pigs offer many important advantages in pharmacological and toxicological research due to its general physiological resemblance to man, but the cost of housing and handling associated with using normally sized pigs is an obstacle to its extensive use as an experimental animal. Consequently, a number of strains of miniature pigs have been developed through selective breeding.

The use of minipigs has thus aroused increased interest during the recent 10 years, as it provides a viable alternative to dogs or nonhuman primates. Particular similarities to humans include its cardio-vascular anatomy and physiology, skin, GI system and digestion, renal system, and immune system. The cost of animals and of housing is comparable to those for beagle dogs (and thus much cheaper than for primates), although some procedures such as dosing and blood sampling may require more manpower, adding slightly to the cost of a toxicity study. A more practical consideration is the fact that minipigs are not specifically discussed in animal welfare laws, as are dogs and monkeys.

The procedures needed to conduct minipig toxicity studies do not differ much from those used for dogs, although naturally some aspects of housing and feeding must be modified. Minipigs may be housed individually or in small male or female groups, but since they are by nature social animals, some contact with other pigs is always necessary. In group housing, the social hierarchy should also be considered (Bollen et al., 1998). Since ad libitum feeding leads to obesity, restricted feeding schedules must be implemented (Ritskes-Hoitinga and Bollen, 1998).

Minipigs are used in all standard preclinical studies: repeat dose, single dose, teratology, fertility assessments, and ADME studies. This also includes all methods of drug administration: oral intubation, dietary, inhalation, dermal including administration to experimental wounds, injection (subcutaneous, intramuscular, intradermal, IV, intraperitoneal, epidural), and continuous IV infusion with ambulatory infusion pumps carried in a jacket.

The most commonly used breeds are Yucatan, Hanford, Sinclair, Hormel, and Göttingen minipigs (Swindle et al., 1994). The information provided in the following will focus on Göttingen minipigs, since this is the main breed employed at Scantox A/S, Denmark, from which data were obtained. Göttingen minipigs are also widely used in Europe and bred in Denmark.

The structure of this section is as follows. In the subsection "General Overview," a general overview is provided, covering systemic findings, an outline of the main steps of the necropsy procedure, including a list of standard organs sampled, followed by a summary of macroscopical and microscopical

findings based on Scantox A/S data. Sections "Cardiovascular System" through "Urinary System" then discuss typical microscopical findings system by system in alphabetical order. For each, our own findings are given, as well as a comparison with humans or other laboratory animals where appropriate.

The data from Scantox A/S are based on findings from more than 150 untreated Göttingen minipigs, used in oral and dermal studies in the period 1997–2001. Ages were from 3 months to 1 year. In the subsection "General Overview," data from 150 of these are furthermore provided in tabular form.

General Overview

Systemic Findings

Göttingen minipigs have been shown to be free of a range of viral, parasitic, fungal, and bacterial diseases (including *Streptococcus suis*) (Hansen et al., 1997; Madsen et al., 2001; www.minipigs.dk).

"Hemorrhagic syndrome" is the common term for an important systemic condition spontaneously occurring in Göttingen minipigs. Its etiology is unknown. In humans, the corresponding syndrome is known as "von Willebrand's disease" and is here an autosomal trait that leads to massive mucosal hemorrhage often resulting in death (Strauss and Bloom, 1965). The syndrome is also seen in the normal pig, which was among the first animal models for this type of genetic hemorrhagic disease (Hogan et al., 1941).

In minipigs, the affected animal usually exhibits poor condition prior to necropsy. The main macroscopical findings consist of generalized multiple petechial hemorrhages in the subcutis of the entire body and in the mesentery. These hemorrhages are easily detected in the mucosal or visceral surfaces of the internal organs, such as the heart, lung, kidney, urinary bladder, and lymph nodes. Further observations may include enlarged pale kidneys, thickened urinary bladder with hemorrhagic mucosa and content, enlarged and reddened lymph nodes, and an edematous thymus. Microscopical findings consist of slight to marked multifocal hemorrhages in various tissues (aorta and aortic arch, heart, kidney, liver, gall bladder, pancreas, lung, lymph node, skeletal muscle, skin/subcutis, stomach and small/large intestines, urinary bladder). These main changes may be accompanied by diffuse hemorrhagic edematous cystitis, interstitial nephritis, hemorrhagic parenchymal necrosis of the liver, reactive lymphoid hyperplasia of the lymph nodes, or increased bone marrow cell density. In surviving animals, hemosiderin deposits can be seen in these organs as remains after hemorrhages.

Necropsy Procedure

Macroscopical examination is performed after opening the abdominal, thoracic, and cranial cavities, respectively. The appearance of the organs is observed in situ.

The standard tissue list (covering the requirement of international guidelines for toxicology studies) includes the adrenal glands, aorta (thoracic), bones (medial condyles of the right femur including knee joint), brain, epididymides, esophagus, eyes with lenses and optic nerves, gallbladder, heart with aortic arch, kidneys, liver (all main lobes), lungs (cranial and caudal lobes), lymph nodes (right mandibular and mesenteric), mammary gland, muscle (right quadriceps femoris), ovaries, pancreas, parathyroids, pituitary, prostate, salivary glands (right parotid and mandibular), sciatic nerve, seminal vesicles, skin, small (duodenum, jejunum, and ileum) and large (cecum, colon, and rectum) intestines, spinal cord (thoracic and lumbar), spleen, sternum (for bone marrow), stomach, testes, thymus, thyroid, tongue, trachea, urinary bladder, uterus (horn and cervix), vagina, and vertebra (thoracic and lumbar). Additional samples are taken from any tissue that appears abnormal macroscopically. Details of any abnormalities are recorded with respect to size, color, texture, and other descriptive characteristics.

Sampled tissues are fixed in neutral phosphate-buffered 4% formaldehyde, except for the eyes (Davidson's fixative) and testes (Bouin's fixative). The lungs are infused with fixative via the trachea. Tissues are processed using standard techniques and embedded in paraffin wax. Sections are cut at a nominal 5 μm and stained with hematoxylin and eosin (H&E). After formaldehyde fixation, osseous tissues are decalcified with acetic acid.

Summary of Macroscopical and Microscopical Data

A summary of the most commonly observed background/incidental macroscopical and microscopical changes at Scantox A/S in the period between 1997 and 2001 is given in Tables 10.6 and 10.7, respectively. Macroscopical data are taken from 124 animals, while the microscopical data are based on 150. All were untreated Göttingen minipigs from 2-, 4-, 13-, 26-, and 52-week oral and dermal studies. As can be seen, spontaneous pathological changes are not very frequent and do not seem to depend on sex or age within the age range studied. They are generally focal and mild in nature.

Table 10.6 Most Common Background/Spontaneously Occurring Macroscopic Changes Observed in 124 Control Male and Female Göttingen Minipigs in 2-, 4-, 13-, 26-, and 52-Week Oral and Dermal Studies between 1997 and 2001

Organ/Finding	Male	Female
Epididymides		
Cyst	1	—
Gallbladder		
Thickened	2	—
Kidney		
Cyst	—	1
Liver		
Marked lobular pattern	—	1
Lung		
Red foci	1	3
Solidifications	1	1
Lymph node (mandibular)		
Enlarged	—	1
Reddened	2	2
Pituitary (pars distalis)		
Red foci	1	—
Small/large intestine		
Red foci	2	1
Spleen		
Gray-white foci	1	—
Submandibular gland		
Edema	—	1
Thymus		
Reddened	4	5
Thyroid		
Red foci	1	3
Urinary bladder		
Reddened mucosa	4	—

Note: Organs are listed alphabetically.

Table 10.7 Most Common Background/Incidentally Occurring Microscopical Changes Observed in 150 Control Male and Female Göttingen Minipigs in 2-, 4-, 13-, 26-, and 52-Week Oral and Dermal Studies between 1997 and 2001

	2 Weeks		4 Weeks		13 Weeks		26 Weeks		52 Weeks	
	M	F	M	F	M	F	M	F	M	F
	7	7	25	25	35	35	4	4	4	4
Cardiovascular system										
Heart										
Mononuclear cells (interstitial, focal) (minimal)	—	—	1	1	—	—	—	—	—	—
Digestive system										
Tongue										
Myositis (focal) (minimal to slight)	2	—	—	5	2	1	1	—	—	2
Ulceration (focal) (slight)	—	—	—	—	1	—	—	—	—	—
Salivary glands										
Mononuclear cells (interstitial, focal) (minimal to slight)	—	—	1	1	3	2	—	—	2	—
Mineralization (glandular, focal) (minimal)	—	—	—	—	—	1	—	—	—	—
Esophagus										
Mononuclear cells (minimal)	1	1	1	—	—	—	—	—	—	1
Stomach (glandular)										
Inflammation (mucosal, focal) (minimal)	1	—	3	2	—	1	—	—	—	—
Erosion (focal) (minimal to slight)	1	—	—	—	—	1	—	1	—	—
Small intestine										
Inflammation (focal/diffuse) (minimal to marked)	5	2	—	—	—	—	—	—	—	—
Peritonitis (focal) (minimal to slight)	—	—	—	—	—	2	—	—	—	—
Large intestine										
Inflammation (focal/diffuse) (minimal to moderate)	4	1	—	—	1	—	—	—	—	—
Arteritis (chronic, focal) (minimal)	—	—	—	—	—	—	—	—	—	1
Liver										
Mononuclear cells (parenchymal/portal) (minimal)	1	2	1	3	4	6	—	—	1	1
Necrosis (parenchymal, focal) (minimal to slight)	—	—	—	1	—	—	—	—	—	1
Single-cell necrosis (minimal)	—	—	—	1	—	—	—	—	—	—
Fibrosis (interlobular) (minimal)	—	—	—	—	1	—	—	—	—	—
Gallbladder										
Cholecystitis (diffuse) (marked)	—	—	—	—	1	—	—	—	—	—
Pancreas										
Mononuclear cells (interstitial, focal) (minimal)	—	—	—	1	1	—	—	—	—	—
Hemorrhage (interstitial) (minimal)	—	—	—	1	—	1	—	—	—	—
Arteritis/periarteritis (necrotizing) (minimal)	—	—	—	—	—	1	—	—	—	—
Endocrine system										
Adrenal glands										
Mononuclear cells (focal) (minimal to slight)	2	—	3	5	4	5	—	—	2	2

(*Continued*)

Table 10.7 (*Continued*) Most Common Background/Incidentally Occurring Microscopical Changes Observed in 150 Control Male and Female Göttingen Minipigs in 2-, 4-, 13-, 26-, and 52-Week Oral and Dermal Studies between 1997 and 2001

	2 Weeks		4 Weeks		13 Weeks		26 Weeks		52 Weeks	
	M	F	M	F	M	F	M	F	M	F
	7	7	25	25	35	35	4	4	4	4
Vacuolation (cytoplasmic, cortical, focal/diffuse) (slight)	—	—	—	—	—	1	—	—	—	—
Accessory cortical tissue	—	—	—	—	—	1	—	—	—	—
Thyroid										
Hemorrhage/inflammation (capsular)	1	2	8	8	10	12	—	1	—	—
Inflammation (interstitial, focal) (minimal to slight)	—	—	—	—	—	3	—	—	—	—
Hematopoietic and lymphatic systems										
Mandibular lymph node										
Sinusoidal hemorrhage (focal/diffuse) (minimal to moderate)	—	2	3	4	6	3	—	—	—	1
Abscess (chronic)	—	—	1	—	2	—	—	1	—	—
Mesenteric lymph node										
Sinusoidal hemorrhage (focal) (minimal)	1	—	—	—	2	1	—	—	—	—
Spleen										
Macrophages (increased) (slight)	—	—	1	—	1	—	—	—	—	—
Arteritis (necrotizing, focal) (minimal)	—	—	—	—	—	1	—	—	—	—
Thymus										
Hemorrhage/inflammation (capsular)	4	5	5	5	8	9	1	1	1	—
Hyperplasia (lymphoid) (moderate)	—	—	—	1	—	—	—	—	—	—
Atrophy (cortical) (slight)	—	—	—	—	1	—	—	—	—	—
Integumentary system										
Skin										
Mononuclear/inflammatory cells (focal) (minimal to moderate)	1	—	1	2	5	8	2	—	—	—
Crust (focal) (minimal to slight)	—	—	—	—	4	6	2	1	—	—
Edema (sub-/epidermal, focal) (minimal to slight)	—	—	1	—	1	6	—	—	—	1
Hyper-/parakeratosis (focal/diffuse) (minimal)	—	—	—	—	3	4	—	—	—	—
Musculoskeletal system										
Skeletal muscle										
Myonecrosis/myositis (focal) (minimal to moderate)	1	1	—	2	3	3	—	1	—	—
Femur										
Serous atrophy of fat cells (minimal to moderate)	1	—	—	—	8	1	2	—	3	—
Tibia										
Serous atrophy of fat cells (minimal tomoderate)	1	—	—	—	7	1	—	—	2	—
Vertebra (lumbar)										
Serous atrophy of fat cells (minimal to moderate)	—	—	—	—	1	—	2	—	—	—

(*Continued*)

Table 10.7 (*Continued*) Most Common Background/Incidentally Occurring Microscopical Changes Observed in 150 Control Male and Female Göttingen Minipigs in 2-, 4-, 13-, 26-, and 52-Week Oral and Dermal Studies between 1997 and 2001

	2 Weeks		4 Weeks		13 Weeks		26 Weeks		52 Weeks	
	M	F	M	F	M	F	M	F	M	F
	7	7	25	25	35	35	4	4	4	4
Nervous system										
Brain (cerebrum)										
Mononuclear cells (meningeal, focal) (minimal)	—	—	1	—	—	1	—	—	—	—
Brain (cerebellum)										
Mineralization (focal) (minimal)	—	—	—	—	2		—	—	—	—
Male reproductive system										
Testes										
Hypoplasia (tubular, focal) (minimal to slight)	3	—	6	—	13	—	2	—	1	—
Granuloma (spermatic) (minimal)	—	—	—	—	1	—	—	—	—	—
Epididymides										
Oligospermia (slight to moderate)	—	—	1	—	1	—	1	—	1	—
Prostate gland										
Mononuclear cells (interstitial, focal) (minimal)	—	—	1	—	1	—	—	—	—	—
Mineralization (glandular, focal) (minimal)	—	—	—	—	1	—	—	—	—	—
Prostatitis (subacute, diffuse) (moderate)	—	—	—	—	1	—	—	—	—	—
Seminal vesicle										
Inflammation (subacute, focal) (minimal)	1	—	—	—	—	—	—	—	—	—
Peri-/arteritis (chronic, focal) (minimal)	—	—	—	—	—	—	—	—	1	—
Female reproductive system										
Ovary										
Mineralization (interstitial, focal) (minimal to slight)	—	2	—	—	—	4	—	—	—	—
Cervix/vagina										
Hypoplasia	—	—	—	—	—	1	—	—	—	—
Respiratory system										
Trachea										
Hemorrhage/inflammation (adventitial)	1	1	1	1	4	1	—	—	—	—
Lung										
Macrophages (alveolar, focal) (minimal to slight)	2	1	6	4	6	7	—	—	—	—
Mononuclear cells (perivascular, focal) (minimal)	2	—	1	1	9	6	—	1	—	—
Mineralization (alveolar, focal) (minimal)	—	—	4	2	1	3	—	—	—	1
Interstitial pneumonia (focal) (minimal)	2	2	—	—	—	2	—	—	—	—
Foreign-body granuloma (focal) (minimal)	—	—	2	2	1	—	—	—	—	1
Hemorrhage (alveolar, focal) (minimal to moderate)	—	—	3	1	—	—	—	—	—	—
Alveolitis (focal) (minimal)	—	—	—	—	—	2	—	—	—	—
Edema (alveolar, focal) (slight)	—	—	1	—	—	—	—	—	—	—
Bronchopneumonia (focal) (moderate)	—	—	—	—	—	—	—	—	1	—

(*Continued*)

Table 10.7 (*Continued*)   Most Common Background/Incidentally Occurring Microscopical Changes Observed in 150 Control Male and Female Göttingen Minipigs in 2-, 4-, 13-, 26-, and 52-Week Oral and Dermal Studies between 1997 and 2001

	2 Weeks		4 Weeks		13 Weeks		26 Weeks		52 Weeks	
	M	F	M	F	M	F	M	F	M	F
	7	7	25	25	35	35	4	4	4	4
Pleura										
Pleuritis (focal) (minimal to slight)	—	—	—	1	1	—	—	—	—	—
Urinary system										
Kidneys										
Mononuclear cells (interstitial, focal) (minimal to moderate)	1	1	6	4	9	13	1	2	1	2
Mineralization (tubular/papillary, focal) (minimal)	—	—	2	—	7	5	—	—	3	2
Tubular basophilia (focal) (minimal to slight)	2	2	2	1	2	6	—	—	—	—
Eosinophils (pelvic, focal) (slight)	—	—	—	—	—	1	—	—	—	—
Casts (tubular, focal) (minimal)	—	—	—	—	1	—	—	—	—	—
Cyst (medullary, focal)	—	—	—	—	—	1	—	—	—	—
Glomerulonephritis (focal) (minimal)	—	—	—	—	1	—	—	—	—	—

Note: Systems are listed alphabetically.

Cardiovascular System

Anatomically, the heart of a pig is similar to a man's, the main exception being the presence of a left azygous vein draining the intercostal system into the coronary sinus (Swindle et al., 1986). The coronary artery's blood supply to the heart is almost identical to that of humans in anatomy and function, and the aorta also has vasa vasorum. As in man, a pig has no collateral vessels in the myocardium (Bloor et al., 1992), leading to increased susceptibility to cardiac infarcts.

All these anatomical parallels have led to the significant interest in using pig hearts for human transplants. The size of an adult human's heart corresponds to that of a pig of between 40 and 50 kg.

As regards handling procedures, the blood vessels and the two atria tend to be more friable in pig than in other species, especially in neonates, and vasospasms are therefore more likely during manipulations. Blood samples for toxicokinetics, hematology, and clinical chemistry are routinely collected from the bijugular trunk near the entry to the thoracic aperture or from the right or left jugular vein. The relative deepness of these blood vessels (Swindle et al., 1988) complicates blood sampling (*cf.* Thyroid Gland).

Pigs play a significant role as an animal model of cardiac disease. The coronary artery can quite simply be clamped, inducing myocardial infarct. Alternatively, administration of a lipid-rich diet will lead to atherosclerosis. In minipig, this is mainly located in the abdominal aorta and in the coronary arteries (Jacobsson, 1989). The Göttingen minipig breed has been demonstrated to be more susceptible to the effects of a lipid-rich diet than the domestic Swedish Landrace (Jacobsson, 1986).

Only minipigs and dogs show cardiac lesions after treatment with vasodilating antihypertensive drugs such as minoxidil (failed attempts include normal pigs, mice, rats, rabbits, and monkeys). Both species develop left ventricular papillary muscle necrosis, myocardial hemorrhage and inflammation, and vascular damage in the atrial epicardium (Herman et al., 1989).

Dogs are also an important model for heart research, but using pigs does provide some important advantages. Minipigs show greater tolerance toward treatment with NSAIDs, antihypertensive

agents, and sympathicomimetic drugs. For sympathicomimetic drugs, in contrast to dogs, no cardiotoxicity is seen in pigs except for an increase in heart rate. Minipigs do not develop arteriopathy with endothelin-receptor antagonists. They are furthermore not prone to vomiting.

Blood Vessels

Arteritis and periarteritis are regularly seen in Göttingen minipigs. One generally sees inflammatory infiltration in all layers of the vessel wall, associated with necrosis and fibrin accumulation (fibrinoid necrosis) (Figure 10.1). Chronic forms can also be observed as thickening of the artery wall caused by fibrotic tissue. Arteritis/periarteritis mainly affects individual arteries of small or medium size and, although generally observed at minimal levels, can occasionally reach moderate. A variety of organs can be affected, in our experience most commonly the epididymides, heart, intestines, kidney, lung, spleen, stomach, and urinary bladder. Similar lesions occur spontaneously in beagle dogs and rats. Kemi et al. (1990) suggested that they can be a result of an immune-mediated mechanism in beagle dogs, while Yu et al. (1982) suggested that food restriction may reduce the incidence of periarteritis in rats.

Also, phlebitis, acute or chronic, is occasionally seen in minipigs. Most commonly, it occurs in the mesenteric vessels.

Heart

Spontaneously occurring cardiac lesions are very rare in Göttingen minipigs. Occasionally, focal mononuclear infiltrates (mainly lymphocytes) can be seen in the interstitial tissue. Necrosis or inflammation of myocardium is seen very occasionally. When found, such lesions appear mild and focal and are characterized by myofiber necrosis associated with inflammatory cell infiltrates.
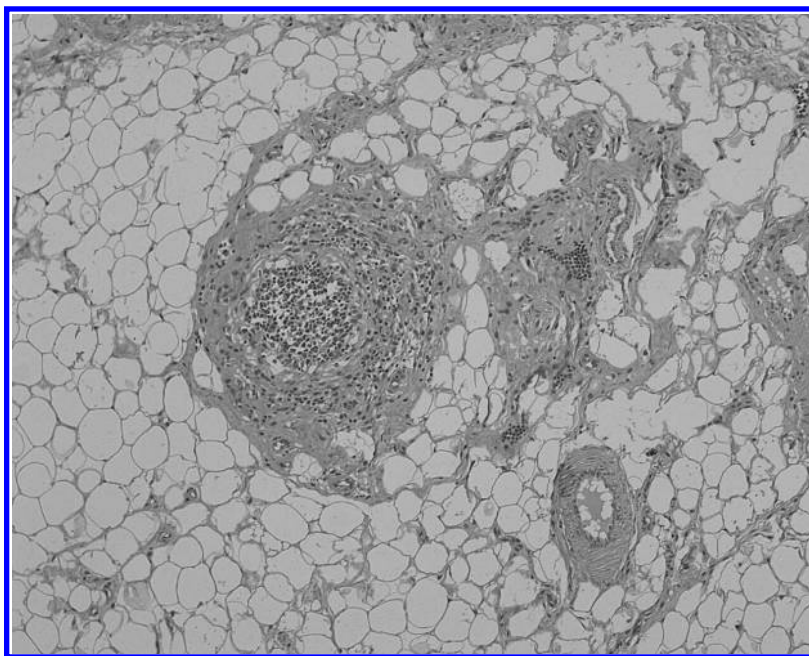
Figure 10.1 Chronic arteritis in a medium-sized artery in the submucosa of the rectum. Inflammatory infiltrate in the lumen and all layers of the vessel wall.

Digestive System

Pigs and humans are both omnivores, and although pigs' digestive system has some anatomical differences from that of humans, the physiology of digestion is nevertheless rather similar. Comparable metabolic functions, intestinal transport times, and nutrient absorption characteristics have made pigs very useful in basic nutritional research (Swindle and Smith, 1998).

Particularly stomach and small intestine have very few differences from humans—pigs have similar gastric cell types, villi, secretions, pH changes, and transit time. The main differences are found in the large intestine—the cecum and colon are somewhat larger in pigs, and the colon is arranged in a series of coils.

Cannulation techniques may be applied both in the GI tract and in the blood circulation allowing the collection of samples of digesta, blood, or tissue.

Tongue

Minimal focal acute or chronic myositis of striated muscle is commonly seen in Göttingen minipigs dosed by oral gavage. The course of this lesion is most likely damage caused by mouth stick inserted into the mouth for introduction of the gastric tube.

Minimal erosive or ulcerative changes at fungiform papilla of nonkeratinized stratified squamous epithelium are very rare.

Salivary Glands

The salivary glands of pigs are large and consist of paired sets of parotid (serous), sublingual (mucous), and mandibular (serous + mucous) glands (Schantz et al., 1996).

Interstitial mononuclear and/or periductular chronic inflammatory infiltrates and mineral deposits within the acini can often be observed at minimal level in Göttingen minipigs.

Periglandular edema of mandibular glands (jellylike material surrounding the gland) is quite a common necropsy finding. The edema is sometimes discernible microscopically in the interlobular tissue.

Minimal focal acinar atrophy of salivary tissue occurs very rarely.

Stomach

Inflammation of the glandular stomach is quite commonly seen in Göttingen minipigs. It is usually minimal, consisting of acute inflammatory infiltrates in the upper lamina propria of the mucosal layer. Erosion is also relatively common at minimal to slight levels, characterized by loss of mucosal epithelium (leaving the basement membrane more or less intact) and associated with acute inflammation in the underlying propria (Figure 10.2). At these levels, the changes can be considered incidental, but treatment may cause higher levels or incidence (Barker et al., 1993). The acute and chronic ulcers that may occasionally be seen consist of tissue damage extending into the deep lamina propria. These lesions in the glandular stomach are usually located in the cardiac and pyloric region and at the duodenal–pyloric junction.

Erosive and ulcerative changes of the nonglandular stomach are occasionally observed, generally at minimal to slight levels.

Small and Large Intestines

The changes occur mainly in the intestinal mucosa. Agonal congestion is very commonly observed, while minimal to moderate focal hemorrhage of the mucosa and submucosa is relatively
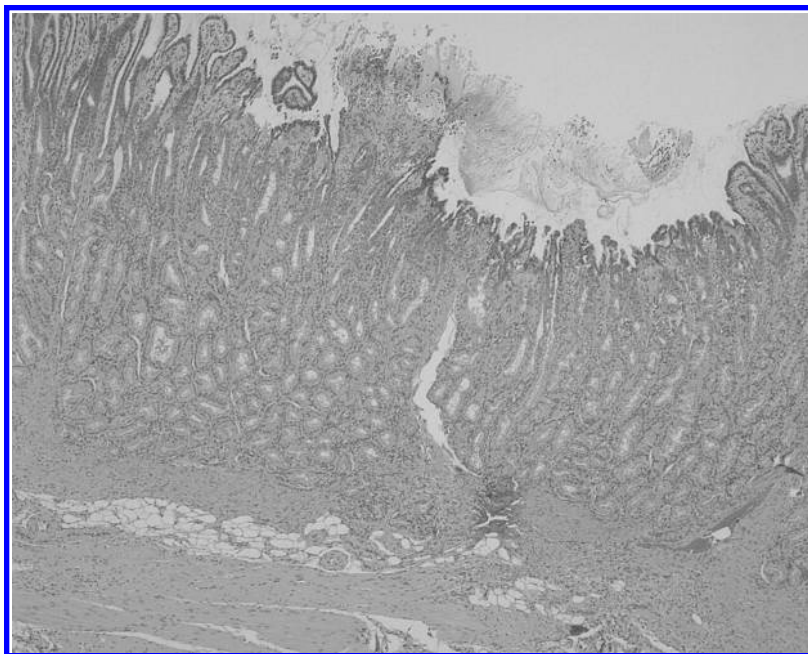
Figure 10.2 Mild focal acute erosion at the pyloric–duodenal junction of glandular stomach. Loss of mucosal epithelium leaving the basement membrane more or less intact.

common, as is focal or diffuse acute and chronic inflammation. Inflammation can reach moderate levels and consists of increased amounts of inflammatory cells in the lamina propria and cellular debris in the glands. It is occasionally associated with erosive or ulcerative changes of the mucosal epithelium.

Proliferative or inflammatory lesions involving the mesentery and the serosal surface of the abdominal organs such as ileum, cecum, and colon are occasionally seen. They can reach moderate levels.

Liver

The liver of minipigs consists of six lobes and is lobulated by rather thick fibrous septa (Schantz et al., 1996). Mildly increased interlobular and/or subcapsular fibrous tissue can occasionally be observed giving some enlargement of stroma and disorganization of the surrounding hepatic parenchyma.

The liver commonly shows focal or multifocal mononuclear cell infiltration, including granulocytes, in the parenchyma and portal areas. It can be associated with single-cell necrosis.

Randomly distributed small foci of parenchymal necrosis can occasionally be seen. The necrotic areas are usually characterized by eosinophilic hepatocytes that have lost cellular detail. They may be associated with inflammatory infiltrates and, more rarely, hemorrhage. In the final stage, fibrotic repair is seen in some cases accompanied by granulomas.

Cytoplasmic vacuolation of hepatocytes due to lipid accumulation can sometimes be observed. These mild focal or multifocal clear, sharp, empty vacuoles usually have no specific zonal pattern.

Clear cell foci characterized by enlarged hepatocytes with clear cytoplasm in the perinuclear region are seen very occasionally in Göttingen minipigs. The clear cytoplasm is a result of glycogen accumulation.

Figure 10.3 Chronic necrotizing cholecystitis in the gallbladder. Note completely necrotized mucosa (upper half of the figure) with underlying fibrosis.

Gallbladder

Chronic necrotizing cholecystitis and hypoplasia and aplasia of the gall bladder are characteristic findings in Göttingen minipigs (Svendsen et al., 1998a).

Of the three, chronic cholecystitis is the one most commonly observed. Macroscopically, the gallbladder is diminished, with thickened walls and thick or absent bile. Microscopically, it is usually characterized by a necrotizing, hemorrhagic mucosal layer with granulomatous inflammation extending into the muscular layer (Figure 10.3).

A hypoplastic gallbladder can be difficult to discern in the macroscopical examination. Microscopically, it appears as loose connective tissue rich in blood vessels, leading to the disappearing of mucosal crypts and flattening of the mucosal epithelium.

Pancreas

The pancreas is rather large in both minipigs and pigs, extending from the spleen to the proximal duodenum. The islet cells are relatively indistinct histologically (Schantz et al., 1996).

Not many lesions occur spontaneously in the pancreas. Small foci of mononuclear cells can sometimes be seen in the interstitial tissue.

Endocrine System

Adrenal Glands

The adrenal glands are located near the cranial poles of both kidneys and the right gland is intimately associated with the wall of the postcava in pigs (Swindle and Smith, 1998).

The most common background finding consists of minimal focal or multifocal mononuclear cell infiltration mainly lymphocytic and usually located in the cortex. In the zona reticularis of the cortex, diffuse cytoplasmic vacuolation can furthermore be seen.

Accessory cortical tissue is observed occasionally. It consists of a portion of the cortex either completely detached from the rest of the gland or attached to it in an enclosing fibrous capsule.

Pituitary Gland

Small cysts can be found in the pars distalis, but are quite rare. They are lined by ciliated columnar epithelium and contain eosinophilic material.

Pars distalis may also occasionally contain mineralized cells, generally randomly distributed and not associated with inflammation.

Thyroid Gland

Accidental mechanical damage of the thyroid gland, or of neighboring organs such as the thymus, esophagus, and trachea, is a common occurrence due to the customary procedure of sampling blood from the bijugular trunk by insertion of a needle (*cf.* Cardiovascular System). The thyroid is located on the ventral surface of the trachea at the thoracic inlet. Such damage consists of capsular and intralobular inflammation characterized by fibrosis, hemorrhage, and macrophages containing pigment (siderin) (Figure 10.4). It can reach marked levels leading to necrosis. Depending on the severity, clinical chemical analysis of thyroid hormone levels may show large variations (Rinke, 1997).

Ultimobranchial cysts can sometimes be seen. They are lined by squamous epithelium and distended by keratin and cellular debris. In rats, such cysts are considered a congenital anomaly (Boorman et al., 1990).
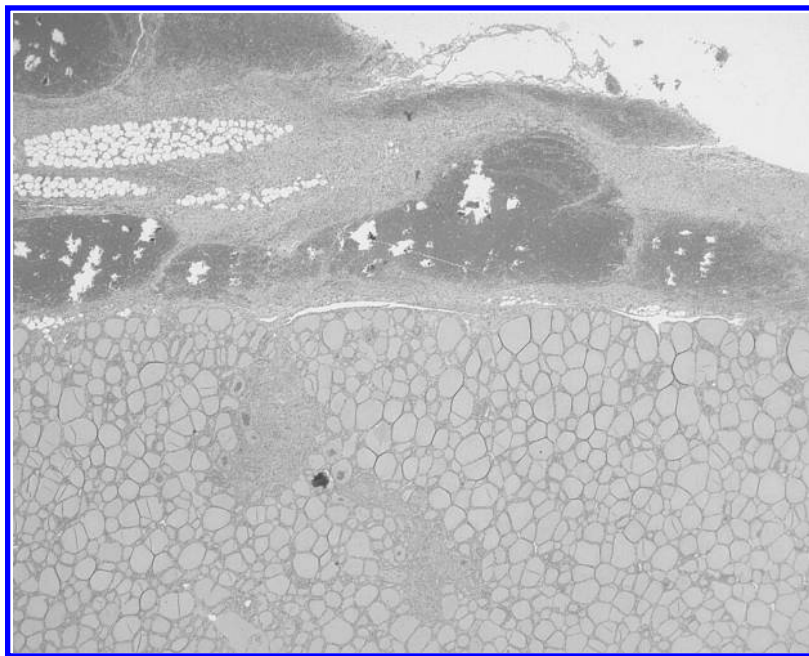
Figure 10.4 Diffuse capsular and intralobular inflammatory, hemorrhagic, and fibrotic changes in the thyroid.

Parathyroid Glands

The pig is the only species without parathyroid glands attached to, or inside, the thyroid gland, and the glands can, therefore, be difficult to locate in necropsies. One can be found on either side of the body, close to the bifurcation of the arteria carotis, embedded in the cranial/middle thymus, fat, or connective tissue.

The Göttingen minipig can show cysts, albeit very rarely. The cysts are usually single and lined by flattened epithelium.

Hematopoietic and Lymphatic Systems

Microscopically, the most frequently observed lesion in Göttingen minipigs is, as in other species, focal mononuclear cell infiltration consisting mainly of lymphocytes with smaller amounts of macrophages and plasma cells (Madsen et al., 1998; Svendsen et al., 1998b). Generally, the infiltration is found interstitially. It may be an indication of a normal immunological potential (Madsen et al., 1998).

Location of iron deposits has been investigated in the Troll minipig (Rinke, 1997) and in the Göttingen minipig (Madsen et al., 1998). They have been found in mononuclear phagocytes (e.g., Kupffer cells in the liver, reticulocytes in the bone marrow, endothelial cells in the adrenocortical sinuses), in mesangial cells in the glomeruli/glomerular tufts in the kidney, and in the sinuses of local lymph nodes. In order to prevent the development of severe anemia during the first 3 weeks of life, newborn piglets are routinely administered colloid iron preparations parenterally, most often in the neck region. The deposits described are remains after this anemia prophylactic injection of a colloid iron preparation shortly after birth (Svendsen et al., 1998b). With age, the deposits may eventually disappear.

Lymph Nodes

The lymph nodes of pigs have a unique histological structure, with centrally located cortical tissue and germinal centers (Figure 10.5) (Nicander et al., 1993).

Routine examinations in toxicological studies generally sample the mandibular and mesenteric lymph nodes. Further investigations may be carried out on the GI and respiratory tracts, as these are of major importance in the lymphatic system (gut-associated lymphoid tissue [GALT] and bronchial-associated lymphoid tissue [BALT], respectively).

Sinusoidal hemorrhage is common in the mandibular lymph node and is likely to be caused by bleeding from needle puncture during blood sampling, but could alternatively be an agonal phenomenon.

Single small abscesses are relatively common in the mandibular lymph node, usually as a result of local irritation or inflammation. In general, they are chronic, characterized by a caseous and calcified center with a surrounding thick layer of fibrotic tissue.

Reactive histiocytosis is relatively common in Göttingen minipig. It is usually minimal and characterized by dilated sinuses containing variable numbers of histiocytic cells. Lymphocytes and plasma cells may also be present.

Hyperplasia of lymph nodes is occasionally observed. It generally refers to increased numbers of lymphoid cells in the germinal centers (follicular hyperplasia) with the overall lymph node structure still preserved. It is common also to see changes in the spleen and in the GALT.

Granulocytic cell series, particularly eosinophils (confirmed by smear preparations), can sometimes be seen—particularly in the mesenteric lymph nodes at minimal level. Their numbers can increase when there is a hemopoietic demand.
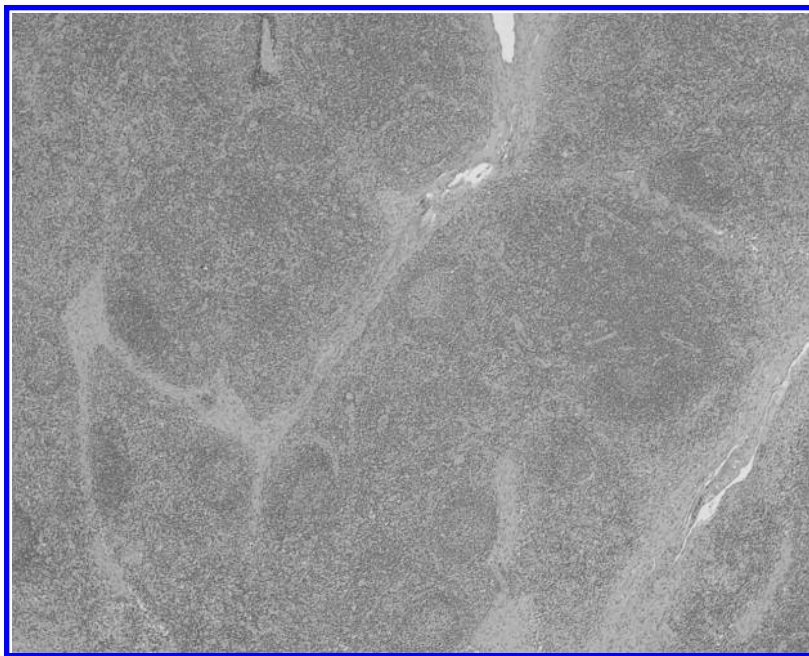
Figure 10.5 Normal structure of a mandibular lymph node in the Göttingen minipig.

Spleen

Minor amounts of cells of the granulocytic series and macrophages are common and difficult to quantify; however, they are described when prominent.

Atrophy of the spleen is very rare as a nonspecific reaction, but it may occur in response to stress or weight loss. It is characterized by lymphocyte depletion of the periarteriolar lymphoid sheaths and marginal zones.

Congestion in the spleen occurs as an agonal phenomenon depending on the mode of death.

Thymus

In minipig, the thymus is located in the cranial thorax and neck lying along the trachea. For that reason, capsular hemorrhage and inflammation are commonly seen due to blood sampling (*cf.* Thyroid Gland).

Cortical atrophy or hypoplasia can occasionally be observed. It involves thinning of the cortex and lymphocyte depletion. Atrophy can be associated with inflammation or stress.

Lymphoid hyperplasia of the medulla can occur very occasionally in Göttingen minipigs. The medulla becomes crowded with lymphocytes, and in advanced cases, the number of germinal centers decreases.

Integumentary System

Pig skin has been shown to be anatomically, physiologically, biochemically, and immunologically similar to humans (Lavker et al., 1991; Zhang and Monteiro-Riviere, 1997) and compares favorably to other laboratory animal species as a model in dermal toxicity studies. Today, approximately one in three pig toxicity studies in this laboratory involves dermal test article administration.

Macroscopically, pig skin, like that of humans, is firmly attached to underlying structures. There is only limited hair cover making pig a very important model for the epithelial healing of split-thickness wounds by the proliferation of hair-follicle epithelial cells (Chvapil and Chvapil, 1992). The surface pH is 6–7, compared to approximately 5 in humans (Meyer, 1996).

Microscopically, most features correspond closely to those in human skin. The epidermis, for example, has the same number of cell layers in the viable zones and in the stratum corneum, and as the only animal, the pig shows the same rete ridge structure as man. Also, epidermal cell membrane glycoproteins (integrins) form a similar pattern to those of human skin (Zhang and Monteiro-Riviere, 1997). Minor differences include the thickness of the epidermis, which is 70–140 μm in pigs compared to 70–120 μm in humans (Meyer et al., 1978), and a dermis, which is slightly more vascularized in humans. Montagna and Yun (1964) have reported dissimilarities between the skin types including a high content of alkaline phosphatase in the epidermis and sebaceous glands of pigs, and the absence of eccrine sweat glands on the body surface of the pig.

Rodents provide an important alternative to the use of minipigs, particularly the albino rat and mouse. After hair removal, any local effect of a test compound on the skin can be evaluated. The skin of rodents is very thin compared to the skin of humans. Nonrodent alternatives are, however, more scarce. Rabbits are not entirely suitable due to the many particularities in their physiology. Beagle dog skin is both hairy and pigmented, making clinical evaluation of local reaction difficult. Some primates constitute suitable models, but their use involves both practical and ethical problems.

The Sinclair minipig strain has proven to be a useful model of the pathogenesis of melanoma growth and regression, due to their disposition for spontaneously occurring cutaneous malignant melanomas and the pathologic similarities to human melanomas (Oxenhandler et al., 1982). Also, the Yucatan strain has proved a useful model in photodermatology studies (Sambuco, 1985). UV radiation was shown to induce sunburn cells in the epidermis, with increasing numbers in response to increased exposure. The response was shown to depend on the suberythemal exposure. These findings agree with the behavior in human and mouse.

Skin and Subcutaneous Tissue

Commonly observed nonspecific histological background findings in Göttingen minipigs include mild focal inflammatory infiltrates, intra- and intercellular edema, crust on the epidermal surface, and acanthosis of epidermis. In dermal studies, the incidence of these changes can be increased due to the method of treatment, e.g., by the mechanical action associated with compound application.

Occasionally, inflammation of the sebaceous glands and hair follicles is seen. Such changes may be associated with nutritional or hormonal factors, as has been suggested for rats (Platt, 1965).

Microabscesses can be seen in the dermis after trauma, in response to foreign material or inflammation.

Musculoskeletal System

The pig is rarely employed in studies of the musculoskeletal system, due to the massive nature of the system in this species and the characteristics of quadruped locomotion (Swindle and Smith, 1998).

It is important to avoid misinterpretation of treatment-related effects with myotoxic potential and parasitic invasion. This is exemplified by severe changes observed in Troll minipigs, in the form of hyaline degeneration and a lymphohistiocytic reaction in the striated skeletal muscles (Rinke, 1997). It occurred in the hind limbs, tongue, and retrotonsillary tissue. Single intracellular sarcosporidia cysts were associated with the affected areas.

Bone

Serous atrophy of bone marrow fat cells is common in the Göttingen minipig (Svendsen et al., 1998b). The degeneration of these cells gives a homogeneous eosinophilic appearance and is generally accompanied by a reduction of the hematopoietic tissue (Figure 10.6). This lesion is classified as minimal or slight, when it is still in the proximal part of the epiphysis of the bone. More rarely, moderate or marked grades are given when the lesion moves to metaphysis and diaphysis. Serous atrophy appears to occur at any age and in either sex, but may be more frequent in males. The precise cause of serous atrophy is unknown, but it occurs in minipigs under normal physiological conditions. Barker (1993) described serous atrophy in calves as a consequence of emaciation and protein–energy malnutrition.

Skeletal Muscle

Minimal to moderate focal or multifocal chronic myositis or myonecrosis is quite common (Figure 10.7). Its focal nature and location suggest that it could be considered nonspecific, possibly a result of trauma (Madsen et al., 1998).

The pig is a good model for studying the local effect of intramuscular injections (Svendsen, 1988).

Nervous System

The porcine brain is relatively large with structures typical of those of other species. The similarities with human brain development and topical, histologic, and vascular anatomy make them useful as general mammalian models (Swindle and Smith, 1998). However, the function of the central nervous system has been relatively little studied. Surgical access to the brain and spinal cord is complicated by the massive nature of the cranial and spinal bone structures.
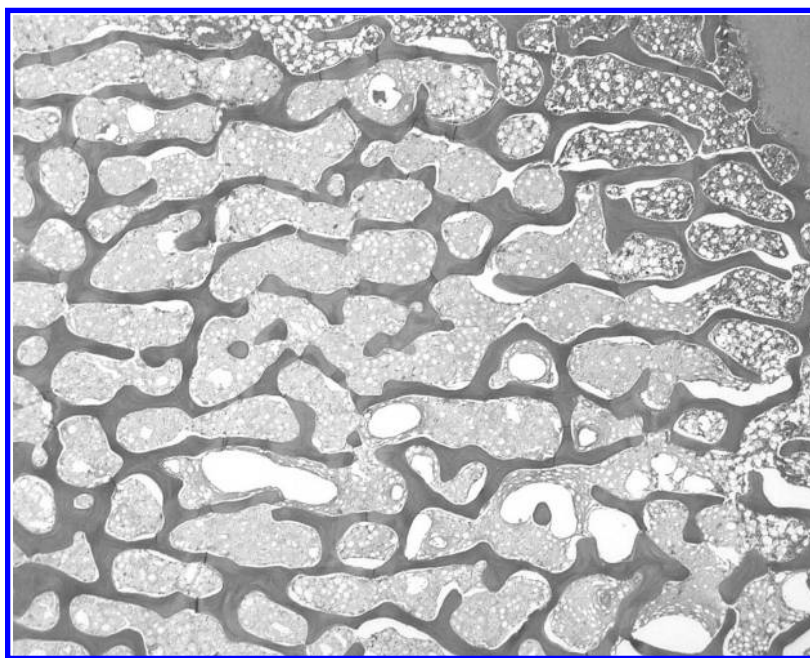
Figure 10.6   Serous atrophy of bone marrow fat cells in the femur.
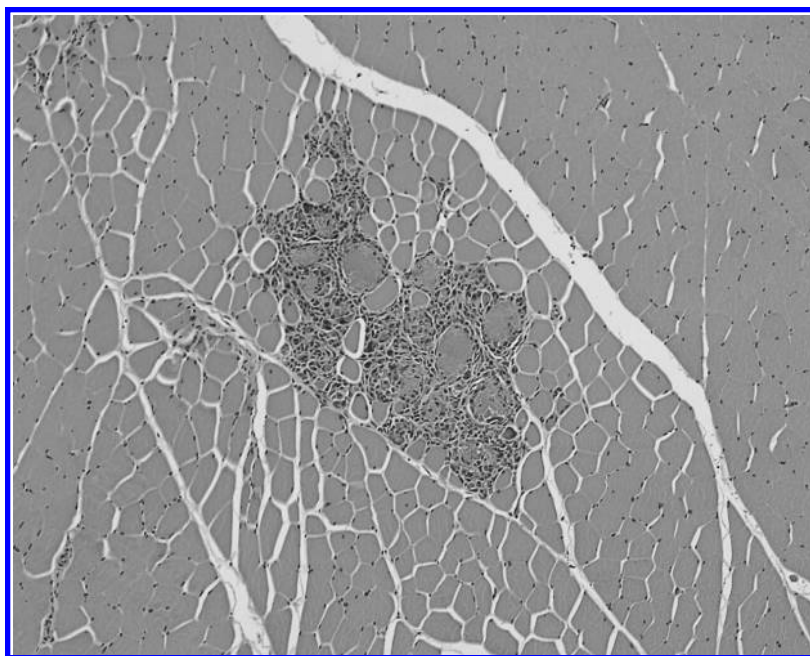
Figure 10.7   Focal myonecrosis of skeletal muscle.

The minipig can be useful in research on Parkinson's disease and provides an economical alternative to primate models (Mikkelsen et al., 1999). In Göttingen minipigs, for example, Parkinson symptoms such as muscle rigidity, hypokinesia, and impaired coordination can be induced. Treatment consists of subcutaneous *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration at a dose of 1 mg/kg/day for 6 days—impaired coordination develops within 5 days.

Brain

Large amounts of mineralized areas particularly in the cerebral/cerebellar leptomeninges are very commonly observed in Göttingen minipigs. Svendsen et al. (1998) mentioned that these mineralized foci are genuine rather than artifacts representing skull bone sawdust.

Also, minimal focal or multifocal mineralized foci located in the pia mater of the meningeal membranes can be seen rather commonly. They are possibly capillaries and their associated pericytes.

Male Reproductive System

The general structure of the male reproductive system is the same as for humans, but the predominant accessory sex glands differ (Swindle and Smith, 1998). They consist of seminal vesicles, the prostate gland, and the bulbourethral glands.

Male Göttingen minipigs reach sexual maturity at 3–4 months (7–9 kg).

Testes

Unilateral or bilateral testicular tubular hypoplasia/atrophy is a common background finding in Göttingen minipigs (Figure 10.8), mainly at minimal levels, but can be up to moderate. Grading is based on the number of hypoplastic tubules. No abnormalities are visible macroscopically.
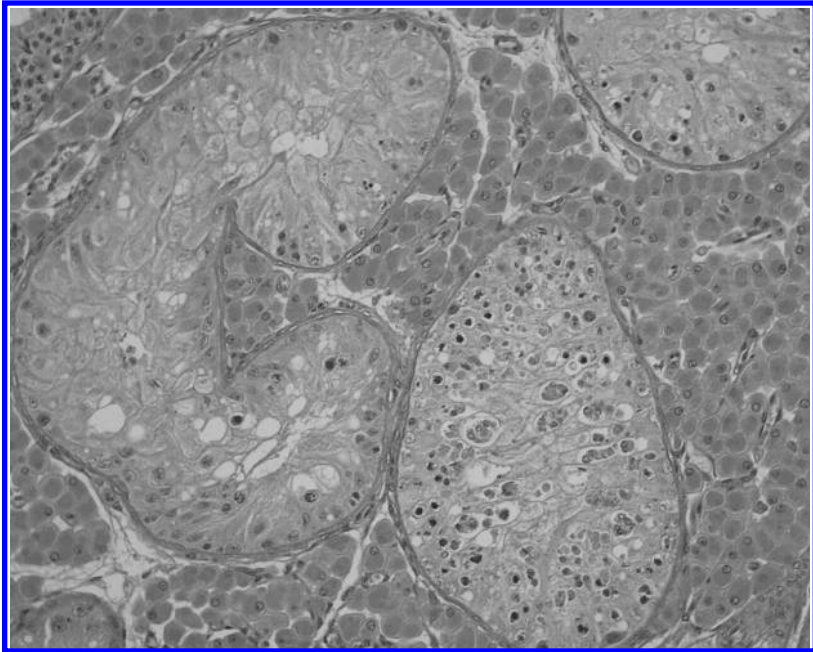
(a)
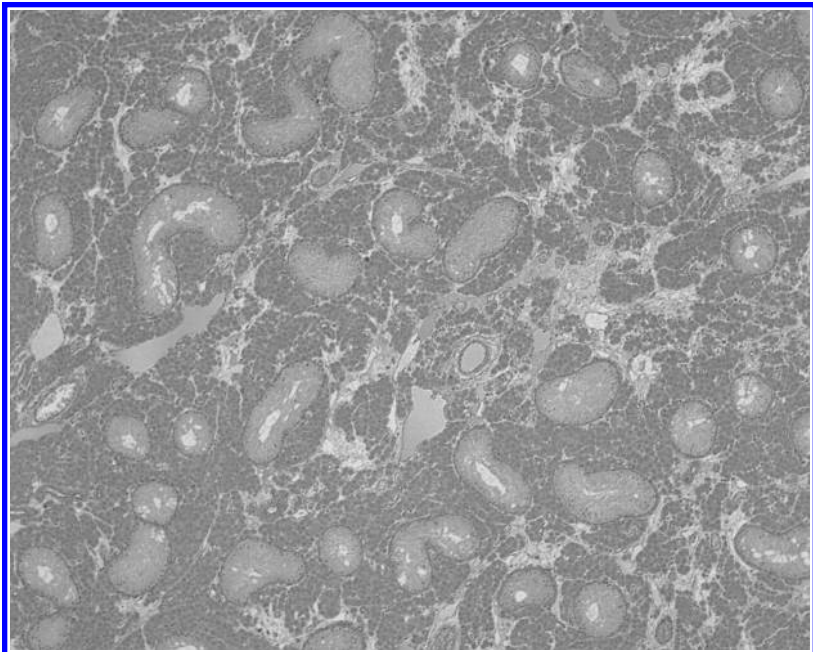
(b)

Figure 10.8 (a) Tubular hypoplasia/atrophy in testes and (b) increased number of interstitial cells (Leydig cells).

The hypoplastic or atrophic tubules are seen microscopically, usually intermingled with normal seminiferous tubules. The abnormal tubules are generally lined by (1) Sertoli cells, (2) Sertoli cells with a basal layer of stem cells, or (3) spermatogonia with no mitotic activity. Their diameters can be diminished, and vacuolation of their germinal epithelium or multinucleate giant cells in their lumen can also be observed.

In moderate cases, the number of interstitial cells (Leydig cells) may seem increased. However, quantitative histological studies in bulls have revealed that the actual volume occupied by the interstitial cells may be less than it appears. Also, in severe cases, concurrent degeneration of Sertoli and interstitial cells was observed. It was proposed that degeneration of Sertoli cells could influence the maintenance and regulation of the interstitial cells (Ladds, 1993).

The cause of testicular tubular hypoplasia/atrophy in Göttingen minipigs is not known. It has been observed in all types of toxicological studies and does not vary with age for (at least) the first year. In bulls, extensive studies indicate a frequency of testicular hypoplasia of the order of 0.5%–1%. It may sometimes be much higher, as in the Swedish Highland breed where an occurrence of 30% has been recorded. This has been shown to be due to genetic factors (Ladds, 1993).

Epididymides

Oligospermia or aspermia is a common finding subsequent to testicular hypoplasia/atrophy in Göttingen minipigs.

Spermatic granulomas occur very rarely in minipig.

Prostate Gland

Small foci of mononuclear cells, predominantly lymphocytes, are frequently found interstitially in the prostate of Göttingen minipigs. Alveolar mineralization is also commonly observed.

Focal minimal to moderate amounts of acute or subacute inflammation in the prostate glands may be seen very occasionally in older Göttingen minipigs. No cause of this was evident; in spite of what could be expected, no involvement of the urinary bladder, ureter, and renal pelvis was observed.

Female Reproductive System

The female reproductive system has a bicornuate uterus with torturous fallopian tubes. The fallopian tubes of an adult female are of the same diameter as those of humans, but much longer. Pigs have an estrous cycle of 20–21 days (Swindle and Smith, 1998).

Female Göttingen minipigs reach sexual maturity at 4–5 months (9–11 kg).

No important background or incidental changes occur in the female reproductive system. In the uterus, focal minimal myometrial hemorrhages and follicular cyst of ovaries can occasionally be seen.

Respiratory System

Functional studies of the airway, including neurochemical anatomy and smooth muscle function, make minipig useful in models of acute respiratory distress syndrome and asthma. The neonatal development of the lungs and airways is useful for extrapolation to humans (Brown and Terris, 1996).

Trachea

Adventitial hemorrhage and inflammation is commonly seen due to blood sampling as explained for thyroid.

Lung

The lungs are composed of apical, middle, and diaphragmatic lobes with an additional accessory lobe for the right lung. The interlobular fissures are incomplete (Swindle and Smith, 1998).

Microscopically, there is quite a lot to note in Göttingen minipigs. However, they are focal and mild lesions.

The most commonly seen in the lungs are multifocal mononuclear infiltrates (predominantly lymphocytes and macrophages) present in the interstitial tissue or perivascularly. Alveolar macrophages are also commonly found (Figure 10.9). Commonly observed, these macrophage populations are regulatory cells controlling inflammatory, immune, and repair processes through the release of a wide array of cytokines and other regulatory molecules.

Mineralized material in alveoli is another common finding, possibly as a result of some debris material.

Interstitial pneumonia (alveolitis) (Figure 10.10) is relatively common. It results from diffuse or patchy damage to the alveolar septa. In Göttingen minipigs, it is generally focal/multifocal, mild, and chronic. The general characterizations of the lesions are alveolar accumulation of mononuclear cells, mostly macrophages, and interstitial thickening by lymphoid infiltrates and fibrous tissue. However, acute form of interstitial pneumonia has been observed. Most interstitial pneumonias in animals are infectious in origin and are caused by viral, bacterial, fungal, or parasitic diseases. Since Göttingen minipigs are free of a range of viral, parasitic, fungal, and bacterial diseases (Hansen et al., 1997; Madsen et al., 2001), these types of condition could be a result of stress factors.

Rinke (1997) has noted that nearly half of their examined animals revealed pneumonic alterations, which were severe in some cases in Troll minipigs. Histologically, they showed the whole spectrum of general lung pathology—even purulent or abscessing components were found. These lesions are due to a mycoplasmic infection, usually secondarily infected by such bacterial agents common in swine livestock as *Haemophilus pleuropneumoniae*, *Bordetella bronchiseptica*,
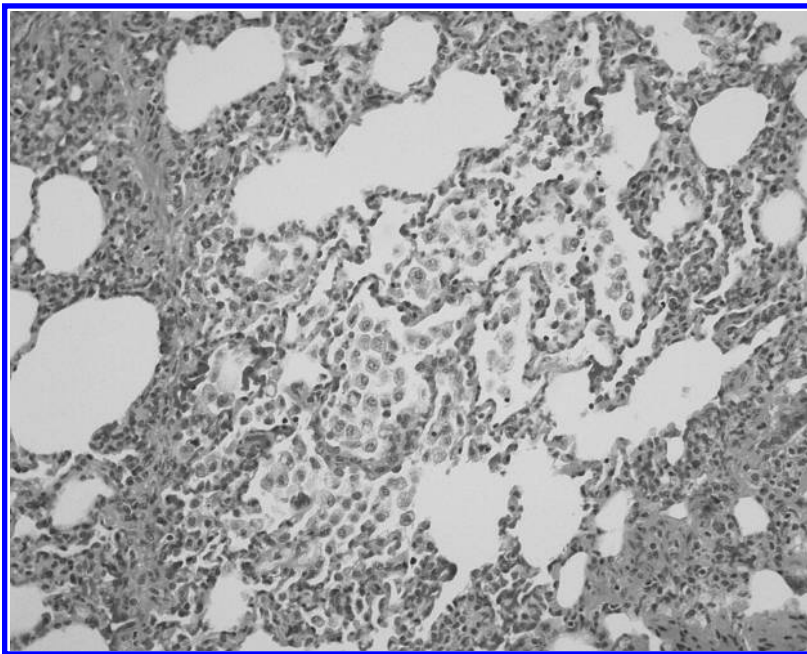
Figure 10.9 Alveolar macrophages in the lung.
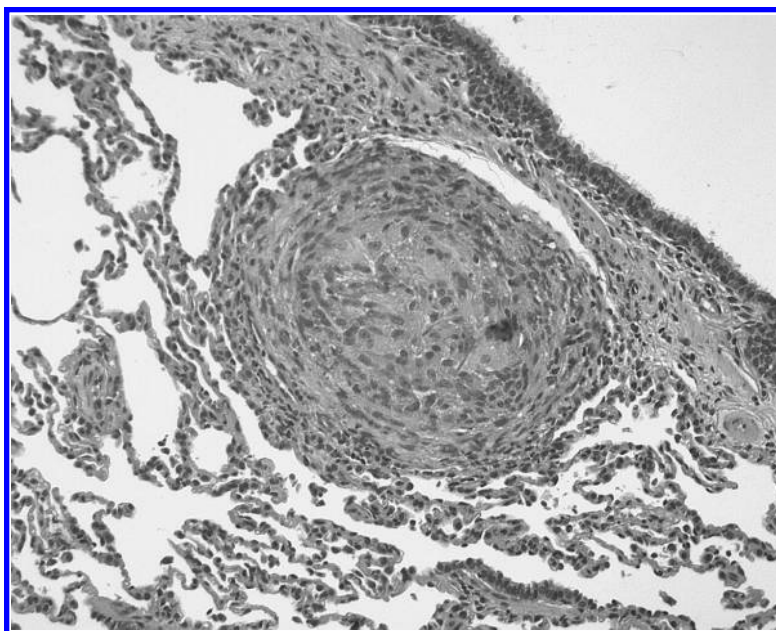
Figure 10.10   An interstitial granuloma in the lung.

and *Pasteurella multocida* (Dungworth, 1993). To avoid such occurrences, optimal hygienic conditions are indispensable.

A variety of foreign-body granulomas can be found fairly commonly in the lungs of minipigs. They are usually hair and particles of diet.

As in the other laboratory animals, congestion and hemorrhage in the lungs are commonly observed agonal phenomena in minipig, related to mode of death.

Arterial thrombus, hematoma, and abscess can be found occasionally.

Pleura

Inflammation of pleural surfaces (pleuritis) is observed occasionally. The inflammation is usually minimal, focal, and chronic. It is characterized by fibrosis and mesothelial cell proliferation. Pleuritis can be associated with the inflammation of the lungs or it could be the result of inflammation extending from the thyroid due to blood sampling.

Urinary System

The pig kidney has anatomical and physiological characteristics resembling those of humans', more than even primates, and is for that reason important in the study of pharmacological agents. As in humans, the pig kidney is multirenculate and multipapillate (Swindle and Smith, 1998).

Kidney

Of the different organs, the kidney most commonly shows focally accumulated mononuclear cell infiltration, mostly minimal, consisting of lymphoid cells in the interstitial tissue.
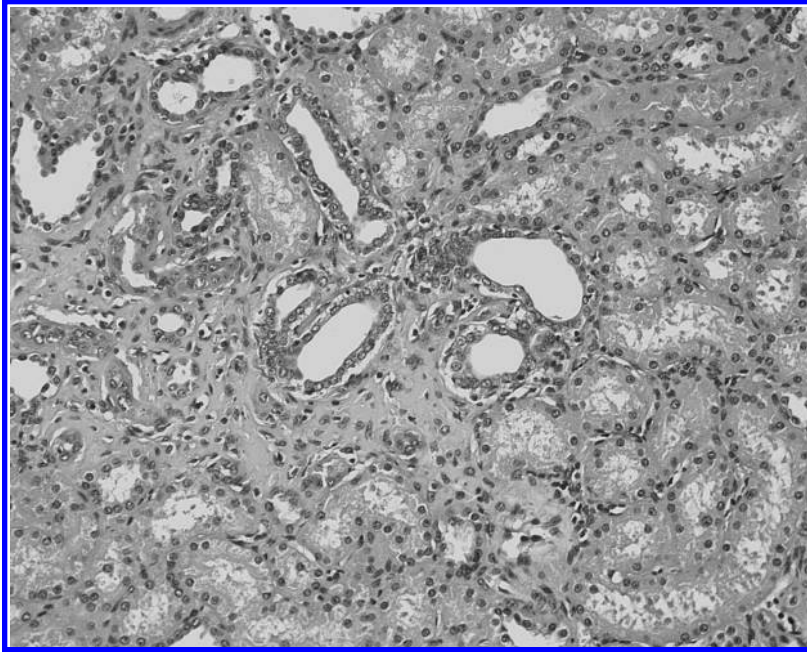
Figure 10.11   Focal area of tubular basophilia in the renal cortex. Dilated tubules with flattened basophilic epithelium and interstitial fibrosis.

Tubular basophilia is regularly observed, particularly in the proximal tubules and mostly in the cortical part of the kidney (Svendsen et al., 1998). It is characterized by basophilic, flattened tubular epithelium sometimes associated with mononuclear cell infiltrates in the surrounding areas. Tubular basophilia is probably the most frequently encountered characteristic of induced nephropathies. It may follow degenerative conditions or represent excessive cell turnover (Gopinath et al., 1987). Because of the focal and mild nature of the change, it is considered to represent de- or regenerating tubules in Göttingen minipigs (Figure 10.11).

Focal mild interstitial inflammation and fibrosis (interstitial nephritis) can be seen on occasion. They are usually located in the renal cortex. These focal and mild lesions can be attributed to tubular defects of resorption or secretion. To see these inflammatory changes at moderate or marked levels is extremely rare.

Small foci of mineralization observed as amorphous basophilic deposits in H&E sections are commonly seen in Göttingen minipig mostly in the papilla.

Solitary cysts and dilated tubules with lumenal proteinaceous casts are occasionally found, usually in the cortex and medulla. They are associated with flattening of the tubular epithelium.

Urinary Bladder

Small foci of inflammatory infiltrates may occasionally be observed in the mucosa and submucosa.

Inflammation of the urinary bladder (cystitis) is observed on rare occasions, secondary to kidney problems.

METABOLISM

Shayne Cox Gad

Minipigs: Metabolism and Pharmacokinetics

Minipigs are increasingly being used in pharmaceutical and toxicological studies; thus, their metabolism has recently attracted considerable attention. Since ingestion is a major route of exposure to foreign substances, significant research involving the absorption and metabolism of these substances is needed. In addition, the effects of foreign substances on normal intestinal functions constitute an area of research requiring further attention. On the practical side, the minipig has significant advantages over the larger farm pig as a model species for evaluating the potential absorption of therapeutics and of their adverse effects. These include logistical issues (relative ease of husbandry and manipulation, reduced need for test materials) and a better degree of characterization of the minipig's systems and absorption and response to xenobiotics.

Comparative structure and function: The GI tract or alimentary canal is a continuous tube that extends from the mouth to the anus through the ventral body cavity. Organs of the GI tract include the mouth, most of the pharynx, the esophagus, the stomach, the small intestine, and the large intestine. The length of the GI tract taken from an adult cadaver is about 9 m (30 ft). In a living person, it is much shorter because the muscles along the walls of GI tract organs are in a state of tonus (sustained contraction). It should be noted that the associated digestive organs are the teeth, tongue, salivary glands, liver, gallbladder, and pancreas. Despite several anatomical differences in the pig, the physiology of digestion remains similar to humans (DeSesso and Williams, 2008).

The buccal epithelium like humans is not keratinized, and domestic pigs have been used for studies on buccal administration of peptides.

Anatomically, the pig stomach is similar to other monogastric species, with the exception of the mucosa. There are two types of mucosa: a nonglandular keratinized mucosa (as in the rat) and a glandular mucosa.

Minipigs are less prone to emesis than dogs, reducing problems of variable or undefined exposure after oral administration of test items. For the dog, vomiting is often a limiting factor in the selection of the species. The minipig is more robust in this respect, but nevertheless, the pig is able to vomit and vomiting may be induced by some test agents. Some mycotoxins (in particular vomitoxin) are noted for provoking emesis in pigs.

The small intestine is long, with a transit time and pH very similar to humans. In a recent study, the mean retention time in the small intestine for 30 kg pigs was estimated to be 4 hours (Wilfart et al., 2007). In the dog, the small intestine is shorter, and the mean small intestinal transit time was determined as 3 hours. Bioavailability of orally administered drugs that are influenced by pH or transit time can be expected to be comparable in humans and pigs.

Pigs show anatomical particularities when compared to dogs, nonhuman primates, and humans, especially in the large intestine where the cecum and colon are larger. The cecum and colon are arranged in a series of coils, known as the spiral colon. The mesenteric vasculature is also different and characterized by a vascular arcade in the subserosa, rather than in the mesentery.

The GI tract contains and processes food from the time it is eaten until it is digested and absorbed or eliminated. In portions of the GI tract, muscular contractions in the wall physically break down the food by repetitive mixing. These contractions also help to dissolve foods by mixing them with fluids secreted in the tract. Enzymes secreted by accessory structures and cells that line the tract break down the food chemically. Wavelike contractions of the smooth muscle in the

Table 10.8   Important GI-Associated Factors for Key Model Species

Humans and minipigs are both omnivores.
Metabolic enzyme systems and function are comparable.
Intestinal transport times are comparable.
Nutrient absorption characteristics are comparable.
Stomach and small intestinal factors have only minimal differences.
Similar gastric cell types.
Similar villi.
Similar secretions.
Similar pH changes.
Similar GI tract transit times.
The minipig and humans have both glandular and nonglandular parts.
Both humans and minipigs are periodic meal feeders.
However, it should be noted that gastric pHs are elevated in young minipigs.

wall of the GI tract propel the food along the tract, from the esophagus to the anus. The digestive system performs six basic processes:

Ingestion: This process involves taking foods and liquids into the mouth (eating).

Secretion: Each day, cells within the walls of the tract and accessory digestive organs secrete a total of about 7 L of water, acid, buffers, and enzymes into the lumen of the tract.

Mixing and propulsion: Alternating contraction and relaxation of smooth muscle in the walls of the GI tract mix food and secretions and propel them toward the anus. This capability of the GI tract to mix and move material along its length is termed motility.

Digestion: Mechanical and chemical processes break down ingested food into small molecules. In mechanical digestion, the teeth cut and grind food before it is swallowed, and the smooth muscles of the stomach and small intestine churn the food. As a result, food molecules become dissolved and thoroughly mixed with digestive enzymes. In chemical digestion, the large carbohydrate, lipid, protein, and nucleic acid molecules in food can be absorbed without metabolism. These include amino acids, cholesterol, glucose, vitamins, minerals, and water.

Absorption: The entrance of ingested and secreted fluid, ions, and the small molecules that are products of digestion into the epithelial cells lining the lumen of the GI tract is called absorption. The absorbed substances pass into blood or lymph and circulate to cells throughout the body.

Defecation: Wastes, indigestible substances, bacteria, and cells sloughed from the lining of the GI tract, and digested material that was not absorbed leaves the body through the anus in a process called defecation. The eliminated material is termed feces.

The wall of the GI tract from the lower esophagus to the anal canal has the same basic, four-layered arrangement of tissues. The four layers of the tract, from deep to superficial, are the mucosa, submucosa, muscularis, and serosa. Mechanisms of absorption of xenobiotics (occurring primarily but not entirely in the intestinal tract) include passive diffusion, active transport, filtration, facilitated diffusion, and pinocytosis. Of total blood flow volume, the stomach (2.6%), small intestines (11.0%), cecum (1.1%), and large intestine (3.4%) collectively receive 18.1% of body blood flow.

Table 10.8 summarizes important GI-associated factors for key model species.

Comparison of Metabolism

Primary metabolism in the GI tract is associated with the P450 enzyme isoenzymes (the CYPs). Table 10.9 provides a comparative summary for which CYPs are involved in the primary metabolism

Table 10.9   Total Cytochrome P450 Content and Cytochrome P450–Mediated Mean Enzyme Activities in Minipigs and Humans

Enzyme Activity	Sex	Göttingen Minipig[a]	Yucatan Micropig[b]	Human[c]
Total cytochrome P450		0.81		0.31
7-Ethoxyresorurfin O-dealkylase activity	F	0.08	0.116	0.03[b]
	M	0.02	0.06	0.09[b]
Coumarin 7-hydroxylase activity	F	0.35	0.97	0.433
	M	0.005	0.18	
Mephenytoin 4-hydroxylase activity		ND	<0.0004	0.032
Debrisoquine 4-hydroxylase activity		ND		
Bufuralol 1-hydroxylase activity		0.72	0.64	0.07[d]
Dextromethorphan O-demethylase activity		4.4		0.125
Chlorzoxazone 6-hydroxylase activity	F	6	0.71	0.216
	M	1.25	0.29	0.216
Testosterone 6-hydroxylase activity		0.87	4.45	1.69
Nifedipine oxidase activity		1.8		0.645[e]

Note: All activities, nmol/mg prot/min; F, female; M, male; ND, not detectable.
[a] Skaanild and Friis (1999) and Skaanild and Friis (2002).
[b] Bogaards et al. (2000).
[c] Chauret et al. (1997).
[d] Smith et al. (1998).
[e] Shimada et al. (1994).

of archetypical substrates. All main metabolic activities typical for human CYP enzymes are found in the minipig liner microsomes.

A different perspective on comparative metabolism is presented in Table 10.10, which looks at key DMPK characteristics for humans and four principal model species.

From these data and the literature, we can make the following statements as to the comparative absorption and metabolism characteristics of minipigs and humans. It is the comparability of these factors that suggests the minipigs as a preferred choice for most orally administered therapeutics.

The reactions catalyzed by biotransforming enzymes are generally divided into two groups, phase-1 and phase-2 reactions. Phase-1 reactions involve the monooxygenases such as cytochrome P450 complex and flavin-containing monooxygenases (FMOs). The cytochrome P450 complex consists of several subfamilies with different substrate specificities, different inhibitors, and inducers. Several of these subfamilies, i.e., CYP1A, CYP2A, CYP2B, CYP2C, CYP2E, and CYP3A,

Table 10.10   Total Body Clearance (L/h/kg) of Some Drug in Minipigs, Dogs, and Humans

Drug	Minipig	Dog	Human	References
Acetaminophen	0.54	0.24	0.3	Bailie et al. (1987)
Vancomycin	0.35	0.18	0.066	Bailie et al. (1987)
Antipyrine	0.64	0.42	0.034	Bailie et al. (1987)
Cefepime	0.12	0.13	0.11	Elkhaili et al. (1997) Gardner and Papich (2001)
Cefpirome	0.11	0.19	0.11	Elkhaili et al. (1997)
Meropenem	0.3	0.24	0.24	Elkhaili et al. (1997) Harrison et al. (1989)
Meloxicam	0.043	0.01	0.01	Busch et al. (1998)
Moxifloxacin	0.645 (1.39)	0.222 (1.33)	0.132 (1.39)	Siefert et al. (1999)

Table 10.11 Minipig Cytochrome P450 Isoenzymes, Substrates, and Inhibitors

Subfamily	Substrates[a,b,c]	Inhibitors[d]
CYP1A	Ethoxyresorufin	Alpha-naphthoflavone
CYP2A	Coumarin	Troleandomycin
		Methoxypsoralen
CYP2B	7-Ethoxy-4-trifluoromethylcoumarin	
CYP2C	Diclofenac	
CYP2D (CYP2B)	Dextromethorphan	Orphenadrine[a]
	Bufuralol	
CYP2E (CYP3A)	Chlorzoxazone	Troleandomycin
		Diethyldithiocarbamate
CYP3A	Testosterone	Ketoconazole
	Nifedipine	

[a] Skaanild and Friis (1999) and Skaanild and Friis (2002).
[b] Anzenbacher et al. (1998).
[c] Bogaards et al. (2000).
[d] Madden et al. (1998).

have been characterized for the minipig during the past few years using hepatocyte microsomes (Anzenbacher et al., 1998; Skaanild and Friis, 1999; Bogaards et al., 2000). Two strains of pigs are commonly used, the Göttingen minipig and the Yucatan mini (micro) pigs. In Table 10.11, total cytochrome P450 and the activity of reactions catalyzed by different cytochrome P450 isoenzymes are presented for both Göttingen minipigs (4 males, 4 females, 4 months) and Yucatan minipigs (3 males, 3 females, 9–10 months). The average total cytochrome for all Göttingen minipigs was more than two times higher than the human values, 0.81 nmol/mg protein (Skaanild and Friis, 1999) versus 0.33 nmol/mg protein (Shimada et al., 1994). Conjugation reactions have long sulfuration.

CYP1A

The O-dealkylase activity of 7-ethoxyresorufin, a known substrate for the CYP1A subfamily, was sex related for both minipig strains. The female contained 2–4 times more than males, whereas the opposite was found for humans (Bogaards et al., 2000) where females contained three times less than males. The activities ranged from 0.02 to 0.12 nmol/mg/min. Anzenbacher et al. (1998) found that the activity in male minipigs was about 7× less than the human activities. Western blotting using antihuman antibodies estimated the apoprotein level for the Göttingen minipig, and a positive correlation between enzyme activity and protein level was found. The mRNA concentration was also measured semiquantitatively using RT-PCR (Skaanild and Friis, 1999). The results from the RT-PCR correlated with neither the activity nor the protein level. This may indicate that the regulation of the expression was translational or pretranslational. Alpha-naphthoflavone, a known inhibitor of CYP1A, strongly inhibited the 7-ethoxypreresorufin O-dealkylase (Madden et al., 1998; Bogaards et al., 2000). The inhibition of CYP1A was stronger in humans (80%–100%) than in minipigs (60%–80%) when incubated with 1 µM α-naphthoflavone. CYP1A2 activity is similar to that in humans and is present in the liver microsome (Preusse and Skaanild, 2012).

CYP2A

The CYP2A activity was determined using coumarin as test substrate. A sex-dependent activity was also seen for the coumarin 7-hydroxylase activities in both minipig strains. Especially, Göttingen minipigs showed differences, the male having 70× lower activity than females (0.005 nmol/mg protein, 0.35 nmol/mg protein) (Skaanild and Friis, 1999). The Yucatan minipigs showed a five times

difference (0.18 nmol/mg protein, 0.97 nmol/mg protein) (Bogaards et al., 2000). The human activity was the same for both sexes (0.433 nmol/mg protein) about the same as the female Göttingen minipig. Again the apoprotein level and the mRNA were determined for the Göttingen minipig (Skaanild and Friis, 1999). Positive correlations were found both between enzyme activity, apoprotein level, and mRNA, indicating a transcriptional regulation of the gene. The activity of CYP2A could be inhibited by both troleandomycin (TAO) and methoxypsoralen, two known inhibitors of CYP2A activity (Madden et al., 1998). Inhibition with antihuman CYP2A6 was strong (60%–80%) indicating that this isoenzyme is the main one responsible for the coumarin 7-hydroxylase activity (Bogaards et al., 2000).

CYP2B

Several CYP2B substrates have been used to measure the activity of this isoenzyme. The 7-pentoxyresorufin O-demethylase and the debrisoquine 4-hydroxylase activities were not present (Anzenbacher et al., 1998; Skaanild and Friis, 1999). However, the 7-ethoxy-4-trifluoromethylcoumarin O-dealkylase activities, another CYP2B-specific activity, were reported in Yucatan minipigs. It was found that females and males contained about the same activity (0.353 nmol/mg protein, 0.286 nmol/mg protein), and they were comparable with the human values. The enzyme activity for both humans and pigs could be inhibited moderately (30%–60%) with anti-rat CYP2B1.

CYP2C

The CYP2C subfamily consists of several enzymes. In humans, four different enzymes have been defined (Guengerich, 1997), CYP2C8, CYP2C9, CYP2C11, and CYP2C19. The diclofenac 4-hydroxylase activity specific for human CYP2C9 was measured in Yucatan minipigs (Bogaards et al., 2000), whereas the mephenytoin 4-hydroxylase activity, a CYP2C19-specific reaction, was estimated in both strains. The diclofenac 4-hydroxylase activities were estimated to 0.06 nmol/min/mg protein for males and 0.02 nmol/mg/min for females, these activities were much smaller than the humans (1.5–1.9 nmL/mg/min), and the activity of the other CYP2C isoenzyme was not detectable. Immunoblotting using anti-rat CYP2C11 did react with the porcine CYP2C protein and the highest protein level was found for the males. The human diclofenac 4-hydroxylase activity can be strongly inhibited (80%–100%) by 10 µM sulfaphenazole, whereas the minipig activity could only be inhibited 15%–30% using the same concentration (Bogaards et al., 2000).

CYP2D

Three different test substrates for CYP2D6 have been used to characterize the minipig CYP2D. Göttingen minipigs possessed no debrisoquine 4-hydroxylase activities, but could biotransform dextromethorphan to dextrorphan. Bufuralol 1-hydroxylase activity was found in both pig strains, but it is questionable whether these activities are catalyzed by the CYP2D isoenzyme family as the reaction cannot be inhibited by either antihuman CYP2D6 or guanidine and quinine, two known chemical inhibitors of CYP2D6 (Bogaards et al., 2000). The dextromethorphan O-demethylase activity could on the other hand be inhibited by orphenadrine a CYP2B inhibitor. The dextromethorphan and bufuralol enzyme activities correlated with the CYP2B protein level in Göttingen minipigs indicating that CYP2B isoenzymes may be responsible for these enzyme activities (Skaanild and Friis, 2002). The CYP2D apoprotein could be measured only using polyclonal antihuman CYP2D6, but not with monoclonal antibody. The CYP2D6 apoprotein concentration did not correlate with enzyme activities.

CYP2E

The chlorzoxazone 6-hydroxylase activity was found to be sex related in both minipig strains, the females possessing the highest activity. In humans, however, no differences between the sexes were seen (Chauret et al., 1997). The Göttingen minipig activity was higher (F, 6 ng/min/mg protein; M, 1.23 ng/min/mg protein) than the activity in Yucatan minipigs (F, 0.771 ng/min/mg protein; M, 0.29 ng/min/mg protein) and humans (0.22 ng/min/mg protein) (Chauret et al., 1997). The apoprotein level and mRNA concentration of CYP2E did not correlate with the enzyme activity indicating that the regulation of the gene expression could take place at all steps in the biosynthesis of the protein or that this substrate is not specific for the minipig CYP2E (Skaanild and Friis, 1999). The human chlorzoxazone 6-hydroxylase activity could be moderately inhibited by anti-rat CYP2E1, whereas the minipig activity was not inhibited. Ketoconazole and TAO, two CYP3A inhibitors, could on the other hand inhibit the activity up to 70%–80% (Madden et al., 1998). This also indicates that chlorzoxazone is not a specific CYP2E substrate in minipigs but a CYP3A substrate.

CYP3A

Testosterone was used as test substrate in both minipig strains, whereas nifedipine was only used when testing the enzyme activity in Göttingen minipigs. Yucatan pigs had a higher enzyme activity than Göttingen minipigs, 4.45 nmol/min/mg protein compared to 0.87 nmol/min/mg protein. The testosterone 6-hydroxylase activity in humans was 1.69 nmol/min/mg protein. The nifedipine oxidase activity correlated well with the testosterone 6-hydroxylase in the minipig, and these activities correlated with the apoprotein level measured using antihuman CYP3A in the immunoblotting assay (Skaanild and Friis, 1999). Ketoconazole, a strong inhibitor of human testosterone hydroxylase, was also a strong inhibitor of minipig testosterone hydroxylation (Madden et al., 1998; Bogaards et al., 2000).

A third strain of pigs, the Hanford minipig, has also been used, but their cytochrome P450 has not been characterized extensively. Peggins et al. (1984) examined the MMFO in a broad range (10 months to 12 years) of Hanford minipigs. They identified definite age-related differences. The amount of cytochrome P450 and the multi-function oxidase (MFO) activity with aniline and p-chloro-N-methylaniline were all significantly higher in middle age (5–8 years) versus young (less than 4 years) minipigs. Freudenthal and Jones (1976) examined Hanford minipigs in the 2–8-month range and obtained somewhat different cytochrome P450 (approximately 0.95 nmol/mg protein) values than did Peggins et al. (1984) (approximately 0.5 nmol/mg protein). This may be compared to the cytochrome P450 values obtained for 4-month-old Göttingen minipig (0.81 nmol/mg protein) examined by Skaanild and Friis (1999). The reported ranges for aniline hydroxylase (about 50 nmol/min/mg protein) were similar in the papers analyzing Hanford minipigs. These results cannot be compared with the two other minipig strains as no values have been reported. Hence, the available data on the MFO of young Hanford minipigs are fairly consistent.

The induction of the different P450 isoenzymes in minipigs has been studied either in vivo or in vitro using liver microsomes or hepatocyte cultures. Early work reporting cytochrome P450 induction characteristics was done by Mueller et al. (1980). They compared the effects of in vivo aroclor induction on the subsequent responses in the Ames *Salmonella* mutagenicity test of seven different species with five different known mutagens. Animals were treated with a single dose of aroclor 1254 (500 mg/kg IP in sesame oil) and sacrificed 5 days later. The minipig ethylmorphine assay responded in the same fashion as rats and mice, with large increases (3.9-fold) in demethylase activity. Liver fraction from untreated minipigs had low activity in the Ames assay with benzo(a)pyrene, cyclophosphamide, and diethylnitrosamine. In contrast, liver preparations from induced animals greatly increased the activity (5–10-fold) in the Ames assay with these mutagens. This is a

pattern very similar to that seen in rats. Thus, the MFO of the minipig is inducible, and the resulting changes in the metabolism may not be dissimilar from those produced by rats. More recent work on induction of cytochrome P450 isoenzymes has been carried out. Kaltenbach et al. (1996) examined the pharmacokinetics of the fungicide itraconazole in Yucatan minipigs (3 females, 1 year). They discovered that the coadministration of rifampin resulted in an 18-fold decrease in the maximum concentration of itraconazole in serum. This was probably caused by an induction of CYP3A activity, as rifampin is a potent inducer of these isoenzymes. In another report, Yucatan minipigs (6 castrates, 6 males, 20 kg) were fed ethanol, 5 g/kg body weight per day, to assess possible links between ethanol-induced oxidant stress, expression of hepatic cytochrome P450, and sex steroid status. The isoenzyme level of CYP2A, CYP2E, and CYP3A was determined using immunohistochemical methods. In control pigs, the CYP2A and CYP3A were more dominant in castrated animals than in males. Ethanol feeding increased the hepatic content of all three CYP isoenzymes. The most significant increase occurred in CYP2E and CYP3A in the males and in CYP2E and CYP2A in the castrated animals. Significant correlations between the levels of the different CYP isoenzymes, protein adducts, and plasma levels of sex steroids were found. The protein adducts were formed between proteins and the metabolites of acetaldehyde, malondialdehyde, and 4-hydroxynonenal. Another report (Stiborova et al., 2001) studied the adduct formation between DNA and metabolized aristolochic acid (AA) for humans, minipigs, and rats using liver microsomes. The DNA adduct profiles were found to be the same for all three species. Further studies using inhibition with alpha-naphthoflavone and furafylline revealed that most of the microsomal activation of AA could be attributed to CYP1A1 and CYP1A2. Inductions of CYP1A and CYP3A in cultured hepatocytes by model inducers have been compared in minipigs (Yucatan, 1 male, 1 female, 15 weeks), humans, Sprague Dawley rats, and beagle dogs (Lu and Li, 2001). Omeprazole was a potent dose-dependent inducer of CYP1A activity in minipigs, humans, and beagle dogs hepatocytes, but not in rat hepatocytes. Dexamethasone and rifampin induced human CYP3A4, with rifampin being the more potent. Conversely, in rat hepatocyte, dexamethasone was a potent CYP3A inducer, while rifampin was not an inducer. Rifampin, but not dexamethasone, induced CYP3A in minipig and beagle dog hepatocytes. These species differences indicate that minipig hepatocytes mimic the human induction pattern better than those of the rat.

The FMO has traditionally been studied in hog liver obtained from slaughter houses (Tynes and Hodgson, 1984; El Amri et al., 1987). Interestingly, when FMO activity is compared between species, substrate specificities are found to be generally very similar (Tynes and Hodgson, 1984). Rettie et al. (1990) have isolated and studied the FMO from Yucatan minipigs. As with the enzyme studied from other species, the hepatic enzyme exists as a single isozymic species, is active with both dimethylaniline (N-oxide formation) and alkyl p-tolyl sulfides (sulfoxidation), and is enantioselective in metabolite formation. It would thus appear that the minipig does not differ significantly from conventional pigs in the presence or activity of FMO.

The phase 2 biotransformation reactions such as conjugations, acetylation, and sulfation have not yet been fully characterized for Göttingen and Yucatan minipigs, but activities in conventional pigs may be used to help infer the expected values until more complete and specific informations appear in the literature on minipigs. However, one of the conjugation reactions, the glucuronosyl transferase using 4-nitrophenol as substrate, was measured in Hanford minipigs and found to be higher in middle-aged (5–8 years) pigs compared to young (less than 4 years) (Peggins et al., 1984). The pig microsomal activity was similar to the activity in rat microsomes, whereas the activity in normal pigs was about double (4.51, 5.5, and 9.38, respectively). The glutathione S-transferase activity in minipig has been compared to the activity in rats. Kirby et al. (1980) reported that ethylene dibromide was rapidly metabolized and cleared by pigs in a fashion similar to that in rats. As ethylene dibromide is a substrate for the rat liver glutathione S-transferase system, that of minipigs must have some characteristic in common with that of rats.

Pharmacokinetics

One of the earliest papers that compare in vivo pharmacokinetic behavior of a specific chemical in the minipig versus another animal studied the toxicology and pharmacokinetics of cyclotrimethylene-trinitramine in rat and minipig (Schneider et al., 1977). Rats convulsed within the first several hours after receiving this chemical, whereas minipigs convulsed at 12–24 hours. This is consistent with the observation that at 24 hours postdosing (100 mg/kg PO), the plasma levels were 3.0 μg/mL in rats and 4.7 μg/mL in minipigs. The latent period for convulsion development was more similar between mini-pigs and humans than between rats and humans indicating that the minipig is a more suitable model for studying human metabolism of nitramines. More detailed reports measuring, i.e., the plasma concentration of drugs, protein binding, and clearance have since been published. Bailie et al. (1987) studied the pharmacokinetics of acetaminophen, vancomycin, and antipyrine in Hanford miniature pigs (5 males, 14–21 kg). On separate days, they were given IV doses of acetaminophen 40 mg/kg, vancomycin 40 mg/kg, and antipyrine 15 mg/kg. Blood samples were collected, and the plasma concentrations versus time profile revealed that the disposition of acetaminophen was monoexponential, whereas the disposition of vancomycin and antipyrine was biexponential. The volume of distribution (Vd) for the three drugs in pigs was the same. Vd for acetaminophen in pigs was smaller than that for humans, whereas Vd for the other two drugs was smaller in humans (Table 10.11). The half-lives of the drug were longer in humans than in pigs especially for antipyrine, and the clearance in pigs was faster than clearance in humans. Again the difference was pronounced for antipyrine with a 20× difference. Cockshott et al. (1992) studied the pharmacokinetics of the anesthetic propofol in rats, dogs, rabbits, and minipigs (4 males, Alderley Park). Following single bolus dose to all animals, blood samples were collected 12–24 hours postdose. The serum concentrations data fitted either a two- or triexponential function depending on the time of sampling. Propofol was distributed into a large initial volume (1–2 L/kg) and extensively redistributed (Vd = 2–10 × body weight) in all species. Clearance by all species was rapid, ranging from about 30–80 mL/kg per min in rats, dogs, and pigs to about 340 mL/kg/min in rabbits. The pharmacokinetics and metabolism of diclofenac, an NSAID, have been studied using four male Yucatan miniature pigs (Oberle et al., 1994). From earlier studies in other animal species, it was known that the metabolism of this drug was species dependent, and large differences between dogs and humans were observed. Compared to human values, the absolute bioavailability of an oral administered buffered solution was high 97%–107% in pigs versus 50% in humans. The total plasma clearance, after IV dosing, in minipigs was 5× slower than in humans (57 ± 17 compared to 252 ± 54 mL/hour/kg), and the plasma level of the different hydroxy metabolites of diclofenac was considerably lower in minipigs. These results suggest slower metabolism and/or enterohepatic recirculation of the parent drug in minipigs indicating that the pig was not an appropriate model for studying the metabolism and pharmacokinetics of diclofenac in humans. In contrast to this, a pharmacokinetic study of the β-lactams cefepime, cefpirome, and meropenem showed that the pig was a better model than rats and monkeys. It was concluded that the minipig was an adequate animal model for studying the pharmacokinetics of these β-lactams (Elkhaili et al., 1997), as $T_{1/2}$, C_{max}, and CL_p were the same for humans and pigs. They also found that it was a reliable model to investigate the influence of the way of administration (direct IV vs. continuous infusion) on tissue penetration of beta-lactams as well as on the dynamics of killing of bacteria. Different administration routes were also studied by Tse and Laplanche (1998). They investigated the role of administration route in absorption, metabolism, and disposition of an acetylcholinesterase inhibitor (SDZ ENA 713). The minipigs (12 males, Göttingen, 4 months, 7.2–8.3 kg) were given a single oral, a single IV, and 2 different dermal doses (3 pigs per group). The oral doses were rapidly and efficiently absorbed (93%), and the bioavailability of the parent drug was low (0.5%) apparently due to extensive first-pass metabolism. The half-life was 56 hours after oral dosing and slightly shorter after IV dosing (48 hours). After dermal administration to the virgin skin, absorption was low (8%), but daily application of placebo patches for 10 days and then dosing

at the same site increased the absorption to 17%–19%. This is probably due to abrasion and hydration of the skin after repeated applications and removal of the adhesive patches. The bioavailability of dermal doses was 20–40 times higher than that of oral doses showing that different administration routes give rise to different pharmacokinetic parameters.

New antibacterial drugs with a broad spectrum are constantly being developed, some of the latest being the 8-methoxyquinolones. The pharmacokinetics of one of these, moxifloxacin, has been compared for humans and other species such as mice, rats, rhesus monkeys, dogs, and minipigs (3 females, Göttingen, 1–2 years, 10–17 kg) (Siefert et al., 1999). The pharmacokinetics was investigated after IV and oral administration of moxifloxacin HCl to animals and human volunteers. The results indicated a clear dependence on the species. Moxifloxacin is absorbed quickly (rat, dog, human > monkey): the major portion of the dose reached the systemic circulation within the first 2 hours. In the minipig, however, absorption was slower. The plasma protein binding was minimal 55%–71% (human > monkey, rat, minipig > mouse > dog); the derived AUC_{norm} was higher in humans than in all-animal species (human >> dog >> minipig, monkey > rat > mouse). Total plasma clearance for all species was higher than that for humans, whereas the renal clearance for the animals was comparable to the renal clearance for humans. The volume of distribution at steady state (Vss) for the animal ranged from 4.9 to 2.7 L/kg monkeys having the highest and dogs the lowest closest to those of humans. Allometric scaling based on body weight and different pharmacokinetic parameters such as Vss, total body clearance, and mean residence time showed a good correlation.

The pharmacokinetics of meloxicam, belonging to another group of drugs, the NSAIDs, was investigated in several species including minipigs (4 males, 13–18 kg, 1 male and 1 female Göttingen minipig, 13 kg), mice, rats, and baboons (Busch et al., 1998). The plasma concentration versus time profiles for meloxicam in rats and dogs was comparable to that of humans, whereas there were marked differences between humans and mice, minipigs, and baboons. The excretion balance on the other hand in minipigs resembled that in humans, with almost equal concentrations being eliminated in the urine and the feces. As in humans, meloxicam circulated mainly in the form of the parent compound in the plasma of mice, rats, dogs, minipigs, and baboons. The main metabolites in rats, minipig, and humans were a 5′-hydroxymethyl derivate and a 5′-carboxy metabolite. The pharmacokinetic profile of meloxicam in rats, however, most closely resembled that of humans with $T_{1/2}$, the same as for humans, whereas the minipig $T_{1/2}$ was about 10× longer (13.4 hours compared to 121 hours).

The pharmacokinetics of two model drugs, atenolol, a beta-blocking agent with small extent of first-pass biotransformation of the parent drug, and 5-aminosalicylic, an antiphlogistic agent with a rapid biotransformation in humans, were compared in humans, dogs, and minipigs (5 Göttingen, average weight 24.5 kg) (Kvetina et al., 1999). The pharmacokinetic parameters, such as $T_{1/2}$ (Table 10.12), AUC, and c_{max}, for atenolol were comparable for humans, dogs, and minipigs. The

Table 10.12   Plasma Elimination Half-Lives (Min) of Some Drug in Minipigs, Dogs, and Humans

Drug	Minipig	Dog	Human	References
Acetaminophen	62	107	120	Bailie et al. (1987)
Vancomycin	88	102	330	Bailie et al. (1987)
Antipyrine	63	78	726	Bailie et al. (1987)
Cefepime	876	65	1080	Elkhaili et al. (1997)
				Gardner and Papich (2001)
Cefpirome	774		1070	Elkhaili et al. (1997)
Meropenem	53	45	50	Elkhaili et al. (1997)
				Harrison et al. (1989)
Meloxicam	6270	144	84	Busch et al. (1998)
Moxifloxacin (PO)	660	540	720	Siefert et al. (1999)
Moxifloxacin (IV)	342	514	780	Siefert et al. (1999)

same parameters for acetylsalicylic acid, however, were not quite the same for the three species. $T_{1/2}$ was about two times longer in humans than in the dogs and minipigs, whereas the AUC was 10 times higher in dogs than in humans and pigs. In humans and minipigs, acetylsalicylic acid was metabolized to N-acetyl-5-aminoacetylsalicylic acid and the pharmacokinetic parameters for this metabolite were comparable in humans and minipigs. This metabolite could not be detected in dogs. This study indicates that for drugs with relatively small "first-pass" effect of biotransformation, there is a greater choice of suitable animal species for studies of comparative pharmacokinetics (as, e.g., in the case of atenolol, where the pharmacokinetic parameters were similar for humans, minipigs, and dogs). On the other hand, when the "first-pass" effect is more pronounced, the situation is more complicated as the choice of the suitable species much more depends on the mechanism of biotransformation of the given drug and on the enzymes of biotransformation. It, therefore, depends on the activity and availability of the biotransforming enzymes in the animal species.

The pharmacokinetics and metabolism of a vitamin D have been studied both in vivo and in vitro using rats and minipigs (3 males and 3 females, 10 kg) and liver microsomes isolated from the two animal species and humans (Kissmeyer and Mortensen, 2000). The pharmacokinetic parameters were the same after one or several doses in the two animal species. A good correlation between the metabolic profile obtained in vitro and in vivo was found, which is important, as most knowledge of the hepatic metabolism in human must be based on the in vitro finding if the drug has not been clinically tested.

Until recently the pharmacokinetics and metabolic studies in minipig have involved oral, dermal, and IV routes of administration. Koch et al. (2001) have investigated the pharmacokinetics of verapamil, a calcium channel blocker, using IV and inhalation as routes of administration. Minipigs (2 males and 2 females, Göttingen) were given an IV injection (0.5 mg/kg) and blood samples were collected. Twelve days after when no verapamil could be detected in the blood, the same animals were exposed for 1 hour to an aerosol concentration of ~50 mg/m^3 verapamil. The total estimated dose after 1 hour was 0.79 mg/kg. The verapamil plasma profile revealed that the pharmacokinetic parameters such as clearance time, initial distribution volume, and partition coefficient, for respiratory delivery of the test substance, are almost the same as for IV administration. The plasma level increased immediately without delay after the start of the exposure, suggesting a fast transfer of the active substance from epithelium into the blood.

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Alternative Species

Shayne Cox Gad

CONTENTS

This book has concentrated on the nine most commonly used laboratory animals. Yet as illustrated in Table 11.1, many other species are used in biomedical research and toxicological assessment that have not been discussed. For example, the chicken is a common model for organophosphate insecticide-induced neurotoxicity (Murphy, 1986), but we have made no mention of the chicken in this book (nor will we again). See Chapter 16 for a more complete discussion on animal model selection.

Discussing all uses for all species used would have resulted in an encyclopedia, not a convenient source book. There are phylogenetic differences in metabolism which reflect evolution (Walket et al., 1978). These less commonly used species, however, should not be totally ignored. The practice of good science demands that a species be appropriate to the question being pursued. The principle of responsible use of animals demands that we consider the replacement of higher animals with lower species (Gad, 1990). Here, we will discuss some less commonly used species that could be used as replacements for other species in some types of toxicity testing. These will be earthworms and fish.

Table 11.1   Example of Uncommonly Used Animal Species

Species	Model or Applications
Armadillo	Leprosy
Aplysia	Behavioral studies
Bear	Sleep/hibernation
Bat	Hearing, sonar, microcirculation
Chicken	Insecticide toxicity, renal excretion
Cat	CNS physiology
Crab	Toxicity screen
Fish	Drug metabolism, carcinogenicity, environmental impact
Hydra	Teratogenic testing
Insects (cricket, fly)	Genetic damage
Japanese quail	Environmental studies
Lobster	Peripheral nerves
Nude mouse	Antitumor drug testing
Owl	Sleep physiology
Pigeon	Behavioral problems
Pig	Surgical models, cardiovascular studies, dermal absorption studies
Salamander	Severed limb regeneration
Sheep	ARDS and other pulmonary phenomenon

EARTHWORMS

Earthworms are invertebrate, cold-blooded animals that collectively belong to the phylum Annelida, class Oligochaeta, and order Megadrili. Earthworms have been one of the more common species used to test chemicals for potential hazardous impact on the environment. The U.S. Food and Drug Administration, for example, includes protocols for the study of earthworms in their *Environmental Assessment Handbook*. As reviewed elsewhere (Chengelis, 1990), earthworms are used for lethality assessment or rankings in place of rodents. Table 11.2 lists common species and sizes of earthworm used in such testing. There is some debate as to which species is best for testing purposes. Some investigators prefer *Lumbricus terrestris* because of its larger size and relative sensitivity (Dean-Ross, 1983). Others prefer *Eisenia foetida* (and the majority of publications mention this species) because of its small size, prevalence in the environment, and relatively low cost (Neuhauser et al., 1986). While there are differences in sensitivity (Neuhauser et al., 1985a), this is a matter of individual preference. The use of *Lumbricus rubellus* has advantages in an urban area because it is easier to obtain, it is still the same size as *E. foetida*, and it is free of the objectionable odor that often accompanies *E. foetida*. See Table 11.3 for a comparison of acute toxicity for various chemicals in *L. rubellus* versus *E. foetida*. In many instances, *L. rubellus* is more sensitive and therefore may be a more appropriate model for lethality screening.

Table 11.2   Earthworm (Phylum Annelida, Class Chaetopoda) Species Commonly Used in Environmental Impact Testing

Species	Common Name	Length (cm)
Allolobophora caliginosa	Field worm	5–20
Eisenia foetida	Manure worm	5–12.5
Lumbricus rubellus	Red worm	5–12.5
Lumbricus terrestris	Night crawler	10–30

Table 11.3 Earthworm Comparative Toxicity

Chemical	LC$_{50}$ (µg/cm^2)	
	Eisenia foetida	*Lumbricus terrestris*
Carbofuran	0.30	0.31
Aldicarb	3.20	0.02
Carbaryl	9.00	0.28
Malathion	13.5	0.27
Parathion	14.8	1.21
Acephate	851	692

Source: Roberts, R. and Dorough, H., *Environ. Toxicol. Chem.*, 3, 67, 1984.

The basic biology of earthworms has been reviewed by Laird and Kroger (1981) and Roberts and Dorough (1985). Worms differ sufficiently from the mammals with which most toxicologists are familiar that a brief review is necessary here.

Earthworms are highly specialized for life in the soil. The outermost barrier of the body is a thin chitinous cuticle, under which is an epidermal layer that contains mucous secretory glands and nerve receptor cells. Some of these are the light receptors that make earthworms photophobic. Locomotion is affected by contractions of the two layers of muscle cells that are underneath the epidermis, a circular layer and a longitudinal layers. The circular muscle layers are responsible for the segmented appearance of earthworms. The central nervous system consists of two ganglia per segment interconnected by a double nerve cord. The circulatory system is a closed-loop system with five hearts and two primary vessels. Respiration is by passive diffusion across the body walls. Earthworms have a coelom, the fluid-filled cavity situated between the body wall from the digestive tract. It permits the worm to crawl in one direction while food is passing through the digestive tract in the other. Interestingly, while hermaphroditic, earthworms do not self-fertilize. They mate with two exchanges of sperm and both partners developing cocoons.

Metabolism

Xenobiotic metabolism has been examined but not thoroughly explored in earthworms. The subject has been reviewed by Stenersen (1984). Because of their size, *L. terrestris* has been the species best studied. It has been shown to have cytochrome P-450-dependent monooxygenase activity, metabolizing aldrin to dieldrin, for example. This highest concentration has been found in the typhlosole, the large fold in the earthworm's intestine. Glutathione *S*-transferase has also been described (Stenersen, 1984). As in mammalian toxicology, it has long been recognized that species differences in toxicity in worms can be due to the differences in metabolism. For example, Gilman and Vardanis (1974) reported that the difference in sensitivity *of L. terrestris* versus *E. foetida* to carbofuran is due to differences in the metabolic distribution of carbofuran.

Husbandry

Caring for earthworms is not difficult (Laird and Kroger, 1981). They literally feed on decaying organic matter found in the soil. One needs only to keep them in moist soil in a cool (15°C–20°C) dark place. Many papers described supplementing this regimen with animal droppings for *E. foetida* (Roberts and Dorough, 1984). It is important that distilled water be used to moisten the soil, as the earthworm can be quite sensitive to organochlorochemicals. For most studies, husbandry procedures are relatively simple. The worms can be kept in the supplier container in a cool dark place and used within 48 hours. Prior to use, worms are rinsed off with distilled water and left in a large petri dish or beaker on a water-loaded filter paper for a few hours in a darkened room. Worms obtained

from domestic bait shops may be of uneven quality and age and should be sorted. Worms obviously smaller, larger, or not as active as the others should not be used.

Dosing Techniques

A variety of dosing techniques have been described in the literature. These include mixing the test article matrices (such as artisol), dipping in aqueous solutions, topical applications, microinjections, and contact on filter paper (Fisher, 1984; Heimbach, 1984; Serda and Furst, 1987).

Microinjection techniques, where the small amounts of test article are injected into the hemocoel or peristrorn, are quite time consuming and can be quite traumatizing (Roberts and Dorough, 1985). Therefore, these techniques are not generally recommended.

Dips in which the worms are placed in a beaker containing a test article solution for 2 hours and then maintained in soil as usual for 1 week have been described (Dean-Ross, 1983). The main disadvantage of this system is that the worms have to be manipulated several times.

Several papers (Heinbach, 1986; Neuhauser et al., 1985b) describe experiments in which the test article is mixed with artificial soils of various compositions in which the worms are then left to reside for 2–4 weeks. This is the method of choice for subchronic studies and is essentially a cross between a dermal application study and a dietary admix study, as the test article will not only be absorbed across the outer cuticle but is also ingested.

All of these routes have very real disadvantages for use in acute lethality testing. With soilborne tests, for example, dead worms will decay and disappear. Hence, one must be prepared to dig frequently through the soil in order to obtain time-to-death estimates. The technique recommended here for this purpose is the filter paper contact method, and this will be the focus of the remainder of this discussion.

Forty-Eight-Hour Contact Test

The 48 hours contact test has proven to be a fast and resource-effective way of assessing acute toxicity of chemicals in earthworms. The fundamentals of this test are outlined on Table 11.4. The standardized method, approved by the European community, is discussed by Neuhauser et al. (1986). This is for environmental impact assessment where cross-laboratory comparisons are important. If, however, one wishes to adopt this technology for the purposes of screening new chemicals, then variants of this method are acceptable, as internal consistency is more important than interlaboratory comparisons. There are two important considerations. First, because of seasonal variation in the quality of earthworms obtained from suppliers, positive controls or comparator chemicals

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Table 11.4   Earthworm 48 h Contact Test Acute Lethality

 1. Place filter paper of known size (9 cm or 12 cm × 6.7 cm) in a petri dish or standard scintillation vial.
 2. Take test article up in acetone or some other volatile solvent.
 3. Slowly and evenly deposit known amounts of test article solution onto filter paper.
 4. Dry thoroughly with air or nitrogen gentle stream.
 5. Add 1 mL of distilled water. Let wet filter paper.
 6. Add worm (*L. rubellus*). Keep in 400–500 mg range.
 7. Ten replicate vials per concentration.
 8. Store/incubate in the absence of light at 15°C–20°C for 48 h.
 9. Examine for lethality (swollen, lack of movement upon warming up to room temp, lack of response to tactile stimulation).
10. Express dose as µg/cm² and mortality as usual. Calculate LC_{50} using standard techniques.
11. Always include negative and positive (benchmark) controls.

should be included on every assay run. Second, distilled water must be used, as worms are quite sensitive to contaminants that may occur in chlorinated water. The filter paper should completely cover the sides of the vessel; otherwise, the worms will simply crawl up the sides to escape the adverse stimulus the chemical contact may provide. General fundamental standards for toxicity testing in nonvertebrates are discussed elsewhere (Anonymous, 1980).

Using these techniques, Robert and Dorough (1984, 1985) and Neuhauser et al. (1986) have compared acute toxicity in a variety of organic chemicals in several earthworm species. A comparison of the lethality of selected insecticides in *E. foetida* and *L. rubellus* has been given in Table 11.3. While there are some obvious quantitative differences between worm species, in general, the rank order of toxicity is about the same. All earthworms are very sensitive to carbofuran under the conditions of this test.

Neuhauser et al. (1985a) have proposed a toxicity rating scheme based on acute lethality in the earthworms, which is similar to the more familiar scheme based on acute lethality in rodents (Table 11.5). Roberts and Dorough (1985) and Neuhauser et al. (1985) have published extensive compilations of acute lethality in worms and compared these to acute lethality in rats and mice. A selection of these is shown in Table 11.6. According to the toxicity rating scheme of Neuhauser, most of these chemicals are in the same toxicity category whether based on results from either *E. foetida* or mice. This may suggest that replacing the LD_{50} with the LC_{50} for rating toxicity (e.g., for a Department of Transportation shipping permit) deserves serious consideration.

To the extent that the 48 hours contact test is artificial in that earthworms are in direct contact with a chemical in a closed system, it may not be truly reflective of toxicity of a chemical to earthworms in the environment. Van Leemput et al. (1989) compared the LC_{50} for enilconazole (a fungicide) in the 48 hours contact test versus a 14-day artificial soil test (OECD guideline 207; protocol summarized on Table 11.7). In the contact test, the LC_{50} was 12.8 $\mu g/cm^2$ (filter paper), whereas in the 14-day artificial soil test, the LC_{50} was 541 $\mu g/g$ (soil). Hence, even given the longer exposure period of the artificial soil test, worms were less sensitive to chemical toxicity in this milieu. The same phenomenon is true for other chemicals (Van Leemput et al. 1989) Thus, while the 48 hours contact test has utility as a predictive, screening, or ranking tool, it has limited value in direct environmental assessment. Additionally (as discussed by Van Gestel et al., 1989), lethality is a poor parameter to

Table 11.5   Earthworm Toxicity Rating

Rating	Designation	Rat LD_{50} (mg/kg)	*Eisenia foetida* LC_{50} ($\mu g/cm^2$)
1	Supertoxic	<5	<1.0
2	Extremely toxic	5–50	1.0–10.0
3	Very toxic	50–500	10–100
4	Moderately toxic	500–5000	100–1000
5	Relatively nontoxic	>5000	>1000

Sources: Neuhauser, E. et al., *Comp. Biochem. Physiol.*, 83C, 197, 1985a; Neuhauser, E. et al., *J. Environ. Qual.*, 14, 383, 1985b.

Table 11.6   Earthworm Acute Lethality Comparative Values

Chemical	*Eisenia foetida* (LC_{50})	Mouse (LD_{50})
2,4-Dinitrophenol	0.6 (1)	45 (2)
Carbaryl	14 (3)	438 (3)
Benzene	75 (3)	4,700 (4)
1,1,1-Trichloroethane	83 (3)	11,240 (5)
Dimethyl phthalate	550 (4)	7,200 (5)

Source: Roberts, B. and Dorough, H., *Environ. Toxicol. Chem.*, 4, 307, 1985.

Table 11.7   Earthworm 14-Day Toxicity Test in Artificial Soil (OECD Guideline 207)

1. Prepare artificial soil; 10% sphagnum peat, 20% kaolinite clay, 69% construction sand, and 1% calcium carbonate (all % by weight).

2. Obtain worms (*E. foetida*) from supplier. Only adults (400–700 mg) with a well-developed clitellurn should be used. Keep for 14 days in shallow trays containing artificial soil before the start of the study.

3. Test article concentrations in test soil are in terms of micrograms per grams of dry weight. Stock solutions in distilled water can be diluted and mixed with soil in a household mixer. Hydrophobic substances can be taken up in a small amount of solvent and mixed with a small amount of test soil. After evaporation of the solvent, the treated soil can be thoroughly mixed with additional soil to obtain appropriate concentrations.

4. For each concentration, four 1 L beakers were filled with 750 g of treated soil. Moisture content was adjusted to 35–40 g H_2O/100 g of soil. Ten worms are added to each beaker. Beakers were covered with perforated plastic.

5. Maintain at 20°C ± 2°C 12/12 h light/dark cycle.

6. After 7 and 14 days, earthworms are removed from the test soil, counted, and sorted. Those not responding to mechanical stimuli are sorted as dead.

7. Mortality data can be analyzed by conventional means.

Source: Van Leemput, L. et al., *Ecotoxicol. Environ. Safety*, 18, 313, 1989.

use in assessing the impact of environmental exposure of earthworms to chemicals. Reproductive function is of greater importance for the maintenance of populations. These authors recommended using cocoon production rather than lethality as a more sensitive endpoint in artificial soil tests. Their reasoning appears to be quite sound. In addition, cocoon counts are easily quantifiable and provide an alternative parameter for assessing toxicity in the artificial soil test where the animals are difficult to see and change in behavior, therefore, is difficult to assess.

Advantages/Disadvantages

The main advantages of the 48 hours contact test are the savings of time and money. The cost savings fall into three categories. First, earthworms are cheap. One hundred *L. rubellus* will cost about $2.00. The 100 mice they could replace in toxicity screens, for example, would cost upward of $500. Second, earthworms require no vivarium space, and their use could decrease the number of rodents used, resulting in a net decreasing in vivarium use. Third, adapting the 48 hours contact test would require little capital investment other than a dedicated under-the-counter refrigerator set at 15°C–20°C. Otherwise, the assay can be easily performed in a standard biochemistry laboratory. With regard to time savings, the standards lethality test with rodents require 7–14 days of postdosing observations. The 48 hours contact test is completed in 48 hours. Not only is the turnaround faster, but the amount of time that technical personnel will have to spend observing animals and recording observations will be decreased. An incidental advantage to earthworms is that they are cold-blooded vertebrates and thus exempt from the requirements of animal welfare laws.

There are two main disadvantages to the use of earthworms in acute toxicity testing. First, there are a limited number of endpoints. Other than death and a few behavioral abnormalities (Drewes et al., 1984; Stenersen, 1979), the test does not yield much qualitative information. Second, there probably is some institutional bias. Because the test is not a cutting-edge technology (no tissue culture) and uses a nonmammalian model, it has been easy to dismiss the utility of the test.

FISH

Fish, like earthworms, have historically been used to assess potential environmental impact. Table 11.8 lists common species of fish and their scientific names. Most fish used in toxicity studies belong to the superclass Gnathostoma, class Osteichthyes, subclass Actinopterygii, infraclass

Table 11.8   Toxicity Testing in Fish Species Studied

Common Name	Formal Name	Use[a]
Bluegill	*Lepomis macrochirus*	E
Carp	*Cyprinus carpio*	M
Channel catfish	*Ictalurus punctatus*	E
Cobra guppy	*Poecilia reticulata*	*C*
Cod	*Gadus morhua*	M
Fathead minnow	*Pimephales promelas*	E
Gulf killifish	*Fundulus grandis*	C
Inland silverside	*Menidia beryllina*	C
Japanese medaka (rice fish)	*Oryzias latipes*	C
Rainbow trout	*Oncorhynchus mykiss* (*Salmo gairdneri*)	E, C, M
Toad fish	*Opsanus tau*	M
Trench	*Tinca tinca*	M
Sheepshead minnow	*Cyprinodon variegatus*	C, E

[a]   E, environmental impact; C, carcinogenicity; M, metabolic studies.

Teleostei, and division Eutelaosti, which include rainbow trout, zebrafish, and Japanese medaka. Rainbow trout in particular have been extensively used in carcinogenicity and mechanistic cancer research (Law, 2003; Stoskopf, 2001). Zebrafish are widely used for a range of endpoints (such as developmental toxicity) as an effective screening model (McCollum et al., 2011; Rubinstein, 2006). They develop most of the major organ systems present in humans (Rubinstein, 2006). They are, of course, cold-blooded vertebrates.

In addition to environmental impact studies, the potential use of fish in carcinogenicity testing has been recognized since the early 1970s. Researchers reported that some fish develop hepatic neoplasias in response to many of the same chemicals that rodents do. In fact, the possible use of fish in carcinogenicity testing was the subject of a National Cancer Institute–sponsored symposium in 1981. This practice, however, has not gained wide acceptance despite data suggesting fish make good models for carcinogenicity testing. Here, the use of the rainbow trout (*Oncorhynchus mykiss*, formerly *Salmo gairdneri*) and the Japanese medaka (*Oryzias latipes*) in carcinogenicity testing will be reviewed.

Husbandry

Fish obviously require water and need to be kept in either glass aquaria or fiber glass or stainless steel tanks. Water should be of consistent quality from a consistent source. Oxygen content, hardness, and alkalinity should be monitored. So long as water quality is consistently monitored and maintained, both the rainbow trout and Japanese medaka are remarkably free of background diseases. Temperature needs to be maintained at 12°C–17°C for trout and 22°C–27°C for medaka. Rates of water turnover in flow-through systems have been described in terms such as 6–10 volume changes per day or 3 L/hour. Published papers describe using aerated well water or dechlorinated tap water. Frequent analysis of potential confounding contaminants should be routine. Lighting can be 12/12 hours cycles. Commercial diets are available for both trout and medaka.

Dosing Techniques

The dosing techniques most often mentioned in the literature include (1) intraperitoneal injection, (2) mixing with water for either static or flow-through exposure, and (3) dietary admix. The intraperitoneal technique is only suitable for treating larger fish and only for single exposures.

Exposure in solution under static conditions requires relatively small amounts of material, is much easier to control, and has been shown to be effective for short-term exposure. Flow-through conditions require relatively large amount of material (and generate large amounts of potentially contaminated water) and require extensive engineering safeguards to prevent the exposure of laboratory personnel to potential carcinogens. Flow-through systems allow for longer-term exposure.

A variety of different systems have been described (see Walker et al., 1985, for an example). Water-based delivery requires a certain level of aqueous solubility that may not always be achievable. In that case, dietary admix is the preferred dosing technique. In fact, dietary admix has proven to be a reliable and effective method for dosing large numbers of fish over long dosing periods and using considerably less test article than flow-through methods. Various methods have been described for preparing the diets, such as dissolving the test article in a highly volatile solvent and applying it by micropipet to individual pellets or mixing it up with salmon oil prior to dressing the basal diet prior to feeding. Exposures are described in terms of parts per million (of test article in the diet). Other dosing techniques have been described in papers in which the emphasis was on studying drug metabolism where relatively small numbers of fish were used in acute preparations. Most of these, such as intra-aortic injection, would be impractical to use for carcinogenicity studies.

Metabolism

Xenobiotic metabolism in medaka has not been extensively examined. This discussion will, therefore, focus on xenobiotic metabolism of the rainbow trout. Some parameters of xenobiotic metabolism are summarized in Table 11.9. Microsomal mixed function oxidase (MMFO) activity has been studied in the rainbow trout in fairly extensive studies since mid-1970 (see Stegman and Kloepper-Sams, 1987; Pesonen et al., 1987, for a review). Trouts have identifiable activity with most

Table 11.9   Rainbow Trout Hepatic Xenobiotic Metabolism

Parameter	Trout	Rat
Liver/body weight ratio (%)	1.1	4.0
Microsomal protein (mg/g)	28.1	25.7
Cytosolic protein	74.5	93.7
Cytochrome P-450	0.34	0.51
MMFO activity		
Benzphetamine	0.2	2.2
Ethylmorphine	0.1	3.8
Ethoxyresorufin	0.4	0.1
Epoxide hydrolase		
Styrene oxide	9.0	6.0
UDP-glucuronosyltransferase		
4-Nitrophenol	1.0	5.0
Testosterone	0.06	0.01
Glutathione *S*-transferase		
Dichloronitrobenzene	5	75
Ethacrynic acid	30	40
N-Acetyltransferase		
2-Aminofluorene	4	0.1
4-Aminobenzoate	0.03	0.06
Temperature (°C)	25	37

Source: Gregus, Z. et al., *Toxicol. Appl. Pharmacol.*, 67, 430, 1983.
Note: Estimated enzyme activities: nmol/min/mg protein.

Table 11.10   Induction of Hepatic Xenobiotic Metabolism in Trout with Aroclor

Parameter	Control (nmol/min/mg)	+PCB[a]
Cytochrome P-450 (nmol/mg)	0.15 ± 0.04	0.29 ± 0.05
MMFO Activity		
Ethoxyresorufin	0.06 ± 0.02	4.07 ± 0.44
Ethoxycoumarin	0.04 ± 0.0	0.49 ± 0.02
Benzo(a)pyrene	0.004 ± 0.006	0.97 ± 0.04

Source: Voss, S. et al., *Arch. Environ. Contain. Toxicol.*, 11, 87, 1982.
[a] Following dietary exposure to 100 ppm Aroclor 1254 (polychlorinated biphenyl) for 5 weeks.

of the substrates that rats do, but the activity tends to be less. No sex- or age-related differences have been discussed in the literature. Gregus et al. (1983) have published comparisons of several of the key enzyme systems in trout to those of the rat (Table 11.9). In most instances, the trout had comparable or even higher activity.

The remarkable aspect of the trout MMFO is the high degree with which it responds to 3-methylcholanthrene (3-MC)-type inducing agents. For example, Voss et al. (1982) reported that 5 days of treatment, by dietary admix with polychlorinated biphenyl (PCB) (100 ppm), resulted in large increases in cytochrome P-450 content as well as increases in 7-ethoxyresorufin, 7-ethoxycoumarin, and benzo(a)pyrene metabolism in rainbow trout (Table 11.10).

Further, Erickson et al. (1988) have reported that exposure of trout to as little as 1 ppm piperonyl butoxide for 3 weeks in a continuous flow-through system resulted a 3-fold increase in cytochrome P-450, a 17-fold increase in ethoxycoumarin demethylase, and a 36-fold increase in ethoxyresorufin demethylase, the latter considered a marker for cytochrome P-448 or 3-MC-type induction.

In fact, because of the extreme responsiveness of trout to this type of induction, Julkunen et al. (1986) have proposed using fish MMFO activity to monitor pollutant levels in a body of water.

Interestingly, trout are not responsive to phenobarbital-type induction (Stegeman and Kloepper-Sams, 1987). For example, Miyauchi (1984) reported that phenobarbital pretreatment did not increase the metabolism or the mutagenicity (in the Ames assay) of 2-acetylaminoflourene, whereas pretreatment with PCBs or 3-MC caused increases in both (Table 11.11). In addition, α-naphthoflavone, an inhibitor of cytochrome P-448 activity, inhibited revertant colony formation, whereas metyrapone, an inhibitor of cytochrome P-450 activity, had no effect on revertant colony formation. Thus, trout differ from rodents with regard to inducibility in both selectivity and degree of response.

Table 11.11   Inaction of Hepatic Xenobiotic Metabolism in Trout (Using the Ames Assay)

	Mutagenicity	
Treatment Group	BENZP[a]	2-AAF[b]
Untreated	20	300
3-Methylcholanthrene	140	600
+ alpha-naphthoflavone	20	90
+ metyrapone	130	500
Phenobarbital	20	290
+ alpha-naphthoflavone	20	20
+ metyrapone	20	220

Source: Miyauchi, M., *Comp. Biochem. Physiol.*, 79C, 363, 1984.
Note: Approximate number of revertant colonies (test strain TA 98) per plate. S-9 fractions from fish treated with either 3-methylcholanthrene or phenobarbital. Inhibitors (α-naphthoflavone or metyrapone) used in vitro (0.1 mm).
[a] BENZP = benzo(a)pyrene.
[b] 2-AAF = 2-acetylaminofluorene.

Trout microsomal enzymes are assayed at room temperature (25°C) or even lower. These are temperatures where MMFO activities in the rat would be severely compromised. The fact that trout have comparable enzyme activities at lower temperature optima than rats is an example of species differences in adaptation. Trout, like all fish, are poikilothermic. How do trout control MMFO activity at different body temperatures? Would small temperature variations affect carcinogen activation? Egaas and Varanasi (1981) compared MMFO activity of rainbow trout kept at either 7°C or 16°C. When benzo(a)pyrene metabolism was examined in vitro at 29°C, the fish kept at the lower temperature had significantly higher activity. Similar results were obtained by Blanck et al. (1988). They compared trout held at 5°C versus 20°C. They observed that both benzo(a)pyrene and ethoxycoumarin metabolism at 18°C in vitro was more rapid in the preparations from the fish maintained at the lower temperature (Table 11.12). However, when in vitro incubations were conducted at the same temperatures at which the fish were kept (i.e., microsomes from fish maintained at 5°C were incubated in vitro at 5°C), there were no differences in enzyme activity.

Hence, the rainbow trout has a mechanism for responding to changes in environmental temperature in order to maintain constant MMFO activity. As noted also in Table 11.12, the different ambient temperatures do not alter cytochrome P-450 content, but do change NADPH: cytochrome C reductase activity. Thus, it would appear that trout respond to changes in ambient temperature by varying the ratio of cytochrome P-450 to the reductase in order to maintain constant MMFO activity. Small variations (group to group in temperature) should not affect carcinogen variation.

Most carcinogens, particularly hepatic carcinogens, require metabolic activation. There are two criteria that should be met to support a conclusion that reactive metabolites are being produced. First, irreversible binding to tissue macromolecules must occur. With regard to the trout, Darnerud et al. (1989) have reported that 1,2-dibromoethane and chloroform form irreversibly bound metabolites in trout exposed in vivo. Egaas and Varanasi (1981) demonstrated that trout had the capacity when assayed in vitro (with S-9 or postmitochondrial supernatant fractions) to catalyze the irreversible binding of benzo(a)pyrene to deoxyribonucleic acid (DNA). Loveland et al. (1988) demonstrated that hepatocytes isolated from rainbow trout were capable of producing metabolites of aflatoxin that irreversibly bound to native DNA. With regard to mutagenic activity, Miyauchi demonstrated that trout hepatic preparations could activate mutagens (2-aminoanthracene and 2-acetylaminofluorene) in the Ames *Salmonella* assay. Thus, it would appear that trout are quite capable of the metabolic activation of carcinogens.

Table 11.12   Hepatic Xenobiotic Metabolism in Trout

Parameter	Ambient Temp. (°C)	Temperature Effects	
		Kept at 20°C	Kept at 5°C
Cytochrome P-450 (nmol/mg)	18	0.10	0.11
NADPH: Cytochrome C reductase (nmol/min/mg)	18	24.9	47.7
Benzo(a)pyrene (pmol/min/mg)	18	17	57
	20	21	—
	5	—	17
Ethoxycoumarin (pmol/min/mg)	18	27	8
	20	37	
	5	—	31

Source: Blanck, J. et al., *Comp. Biochem. Physiol.*, 93C, 55, 1989.
Note: Microsomal enzyme activities in vivo from mature rainbow trout. Water temperatures were recorded at the time of harvest. N = 15–20.

Examples of Carcinogenicity in Fish

Numerous reports of chemical carcinogenicity in fish exist and only a few illustrative reports will be presented here. Hendricks et al. (1985) studied the carcinogenicity of benzo(a)pyrene in rainbow trout. The study was initiated with 3-month-old trout fingerlings (3.3 g). They were continuously fed a dietary admixture containing 1000 ppm benzo(a)pyrene for 18 months. The results are summarized on Table 11.13. While there was no difference in mortality, there were decreases in body weight, increases in liver to body weight ratio, and a significant increase in hepatic carcinomas. Thus, benzo(a)pyrene is a positive hepatic carcinogen in rainbow trout. There were no hepatic neoplasias in the control group, which is a common observation (e.g., very low tumor background rate) in carcinogenicity studies in fish.

Shelton et al. (1984) reported that diethylnitrosamine (100 ppm by dietary admix) produced liver tumors in trout with 12 months of treatment and that cotreatment with PCBs (MMFO-inducing agents) greatly enhance the tumor yield (Table 11.14).

Hawkins and Hinton and their colleagues have reported on the carcinogenicity of methylazoxymethanol (Hinton et al., 1984) and benzo(a)pyrene (Hawkins et al., 1988) in medaka (Table 11.15). They have also identified tumors in medaka in organs other than the liver. (For a more complete review of carcinogenicity testing with medaka and other aquarium fish, see Hawkins et al., 1985.) Benzo(a)pyrene, diethylnitrosamine, and methylazoxymethanol are presumed, if not confirmed, human carcinogens. Thus, it would appear that rainbow trout and Japanese medaka are viable species in which to test suspect human hepatic carcinogens.

Currently, fish toxicity testing is mainly based on in vivo tests. The in vivo tests are of ethical concern, and they suffer from technical limitations and low cost–benefit efficiency. Their ecotoxicological relevance is doubtful, since they are not able to detect a number of relevant toxic effects.

Table 11.13   Rainbow Trout Carcinogenicity Study

	Control	BP
N	114	114
Deaths	5	3
Body weights (g)	425 ± 153	364 ± 125
Liver/body weight (%)	0.66 ± 0.16	0.70 ± 0.13
Liver neoplasia		
Altered hepatic foci (%)	0	4.5[a]
Carcinoma (%)	0	21

Source: Hendricks, J. et al., *J. Nat. Cancer Inst.*, 74, 839, 1985.
Note: Study started with 3-month-old trout fingerlings (3.3 g), fed 100 ppm benzo(a)pyrene (BP) by dietary admixture ad libitum for 18 months.
[a] Incidence of animals having the lesion.

Table 11.14   Rainbow Trout Carcinogenicity Study

	Control	DEN	DEN + AC
N	120	120	120
Deaths	1	2	28
Liver row	0.77 ± 0.08	0.65 ± 0.11	0.89 ± 0.13
Liver neoplasia (%)	0	10.2	40.2

Source: Shelton, D. et al., *Toxicol. Lett.*, 22, 27, 1984.
Note: Trout fingerlings fed 1100 ppm diethylnitrosamine (DEN) or DEN + 100 ppm Aroclor (AC) 1242, by dietary admixture for 12 months.

Table 11.15   Medaka Carcinogenicity Study

	Incidence of Hepatic Tumors	
Dosage Groups	**At 24 Weeks**	**At 36 Weeks**
Negative control	0/82	1/89 (~1.1%)
Solvent control	1/75	1/97 (~1.0%)
1–4 PPB	1/75	1/94 (~1.1%)
8–34 PPB	0/70	0/96 (0%)
200–220 PPB	8/76	26/73 (~36%)[a]

Source: Hawkins, W. et al., *Ecotoxicol. Environ. Safety*, 16, 219, 1988.
Note: Initiated with two 6 h exposure periods to benzo(a)pyrene, 6 days apart when fish were 6–10 days of age. Maintained for 36 weeks under standard aquarium conditions.
[a]   Twenty had adenomas and six had carcinomas of the liver.

To date, the use of fish cells in ecotoxicology has mainly been focused on the measurement of (a) cytotoxicity, both basal and selective (cell specific); (b) genotoxicity; and (c) effects on cell-specific functions and parameters, including studies on biotransformation and the induction of biomarkers or mechanisms of toxicity. In vitro systems based on fish cells have been found to be valuable:

For the toxicity ranking and classification of chemicals, including quantitative structure–activity relationship studies: These types of studies are largely based on the measurement of basal cytotoxicity, where fish cells perform as well as mammalian cell systems. The more practical handling of fish cells even favors the replacement of mammalian cells for some purposes.

For toxicity measurements on environmental samples: For this type of sample, fish cells offer a number of technical advantages over mammalian cells, as well as over in vivo fish tests, particularly for bioassay-directed fractionation studies, which are of increasing importance in environmental toxicology. The rapid, differentiated effects assessments provided by fish cells, as well as their need for only small sample volumes, represent distinct advantages over in vivo assays.

For toxicity characterization of chemicals and environmental samples: Fish cells in vitro provide a bioanalytical tool for the assessment of differentiated effects and the possibility of screening in a rapid and cost-effective way, for a wide range of endpoints (for example, cytotoxicity, genotoxicity, dioxin-like activity, endocrine disruptor activity). Fish cells can be used to develop relevant ecotoxicological endpoints (for example, the prediction of genotoxic effects in terms of genetic diversity). The introduction of genomics and proteomics will further enhance the potential value of fish in vitro systems.

For studies on toxic modes of action: Fish cells in vitro can address aspects of toxic mechanisms that are difficult to study in vivo; for example, chemical-induced receptor activation, attacks on the permeability properties of the gills, or temperature effects. A further advantage of the in vitro system is that it is based on a relatively uniform cell type. Compared to mammalian cells, fish cells are the models of choice for investigations on fish-specific toxicokinetic and toxicodynamic processes.

The reduction and complementation of in vivo ecotoxicity tests by in vitro assays could greatly improve the quality of ecotoxicological hazard assessments. The in vitro systems offer the possibility of the more differentiated assessment of effects, involving the use of more endpoints, and could provide the necessary mechanistic understanding for effect prediction and the systematic classification of chemicals.

However, a disadvantage of fish cell systems, which they share with other cell systems, is their lower absolute sensitivity compared to the fish used in in vivo tests. The absolute sensitivity of tests is of relevance, for example, in the determination of environmentally safe levels of chemicals. This, however, is usually not done on the basis of a single test, but the results from several tests. Extensive data are available to support this investigation (Castano et al., 2002).

Advantages and Disadvantages

There are three main advantages to using fish in carcinogenicity testing. First, they have an extremely low background tumor rate, which enhances the sensitivity of the assays. Second, fish are less expensive to purchase and maintain than rodents. Group sizes of 100 or more (particularly with medaka) become quite manageable. Third, a positive carcinogen will generally show up in fish within 1 year's time. Rodent studies generally last for 18 months (mice) to 30 months (rats). Medaka have certain advantages over trout that magnify the cost savings. For example, they will thrive in waters maintained at room temperature, whereas the cooler temperature optimum of the rainbow trout generally requires the use of energy-dependent cooling systems. The larger size of the trout requires greater aquarium space. The smaller size of the medaka is an advantage at necropsy, where medaka are fixed and slide mounted in toto for histological examination, saving both time spent on dissection and other histology laboratory resources.

There are three main disadvantages in using fish for carcinogenicity testing. First, compared to rodents, only relatively few tumors, primarily carcinomas of the liver, have been described in fish. Hence, it would appear to be prudent to use carcinogenicity testing in fish as an adjunct to testing in at least one mammalian species. On the basis of current data, fish would be best used in confirming potential hepatic carcinogens. Second, the assays cannot be adapted without capital changes to the standard vivarium. Third, as with earthworms, there is an ill-defined institutionalized bias against nonmammalian animal models. One could argue, however, that a chemical shown to be carcinogenic across a broad phylogenetic spectrum of species is a stronger candidate to be a human carcinogen than one shown to be positive in only one sex or species.

CATS

While their use is limited due to political sensitivities, the domestic cat is the appropriate model in evaluating the drugs and vaccines for use in the cat as a companion animal and in some specific cases where their metabolism is the best model for that in humans (Gupta, 2012; Peterson and Talcott, 2012). Some excellent cardiovascular studies have been recently published (Taylor et al., 2012), providing an alternative means of evaluating adverse pharmacological effects. They also have historically been a preferred model for laryngeal irritation.

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Clinical Pathology of Laboratory Animals

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CONTENTS

In conventional toxicology studies, clinical pathology usually consists of routine hematology, clinical chemistry, and urinalysis tests. The majority of tests are the same as those used in human or veterinary medicine to establish a minimum database. There are, of course, species differences for references ranges, interpretation of changes, methodology, and the value or appropriateness of individual tests. The choice of tests for a given study depends upon a number of factors including objective of the study, test species, test material(s), duration of the study, and regulatory requirements.

Clinical pathology test results are routinely used to identify general metabolic and pathological processes. Since multiple collections are usually possible for clinical pathology samples, they allow monitoring of effects overtime to see progression of changes or pharmacodynamic end points. Also, clinical pathology tests may be more sensitive than histopathology or clinical observations in detecting injury in certain organs such as bone marrow. However, specific "diagnoses" or toxicologic mechanisms are infrequently identified and clinical pathology test results are rarely the only evidence of biologically important *adverse* toxicologic effects. Clinical observations and/or anatomic pathology findings usually corroborate pathologically meaningful laboratory findings.

On the other hand, it is relatively common to observe minor effects of a test material only in the clinical pathology results. For example, changes in homeostatic mechanisms to maintain normal fluid, electrolyte, and acid–base balance secondary to a test material that alters water consumption or acid load may be reflected only in urinalysis results.

The ability to identify subtle changes in a group of treated animals that are compared directly with a control group is much greater than in an individual animal compared with historical reference ranges. In safety assessment studies, this fact inevitably leads to important decisions concerning the nature of a subtle change. Is it simply an alteration in normal homeostatic mechanisms or is it an early indication of a more significant adverse effect? This question can be difficult to answer. It is clearly not as simple as referring to a table of historical reference ranges.

Accurate interpretation of laboratory results requires not only an understanding of the tests themselves but also knowledge of species differences, study design and procedures, the test material(s), clinical observations, and anatomic pathology findings. The interpretation of one test result, e.g., high serum urea nitrogen, is dependent on the results of another, i.e., urine specific gravity. The urine specific gravity result is the difference between an interpretation of prerenal azotemia, as with dehydration, and renal azotemia, as with renal failure.

With respect to regulated safety studies, required or recommended clinical pathology tests have important limitations in their ability to demonstrate toxicologic effects. Some of the limitations are due to timing of sample collection and analysis during the study, and some are related more to the actual tests. In fact, some of the recommended tests are outdated or inappropriate and should be either eliminated or used only in specific circumstances. To add to the confusion, recommended test lists vary among regulatory agencies, both inside and outside of the United States.

This chapter will address (1) the use and potential misuse of clinical pathology reference ranges; (2) sources of variation in laboratory test results and their potential impact on data interpretation; (3) the characteristics and interpretation of routine hematology, clinical chemistry, and urinalysis tests used in toxicology studies; and (4) aspects of laboratory test results that are unique to individual laboratory animal species. For more in-depth discussions of the laboratory tests and their interpretation, the reader is referred to more additional texts by Kaneko et al. (2008), Latimer (2011), Loeb and Quimby (1999), Suckow et al. (2012), Stockham and Scott (2008), Thrall et al. (2004), and Weiss and Wardrop (2010).

Diagnostic terminology has been used in this chapter to discuss abnormal test results; however, use of such diagnostic terms is not preferred in toxicology studies because they represent a disease condition, whereas changes identified in the context of toxicity study are not always of the magnitude to represent a disease condition.

REFERENCE RANGES

The term "normal range" has been replaced by the more appropriate terms "reference range" or "reference interval." The word "normal" occasionally caused confusion because it implied that values outside of the range were, by definition, abnormal. However, because most laboratory test reference intervals are statistically constructed to include the range of values found in 95% of a population of healthy, or "normal," individuals, it should not be surprising that approximately 1 out of 20 results for a specific test (e.g., serum glucose concentration) from a group of "normal" animals is outside of the historical reference range. Taking this idea a step further, and because of the fact that a minimum hematology and clinical chemistry database consists of approximately 30 individual test results, it should also not be surprising that a "normal" animal will often have at least 1 test result that falls outside of the historical reference ranges. In this light, the idea that all study animals have "normal" clinical pathology test results seems misconstrued.

Another potentially confusing aspect of the term "normal range" is that it seems to imply that a test result within the range is, by definition, normal, and therefore the organ system or metabolic process being assessed by that test is also normal. Unfortunately, severely abnormal animals may have laboratory values well within the reference range. For example, a dog with advanced liver cirrhosis often has serum liver enzyme activities within the established reference ranges for those tests. An animal with leukemia may have a "normal" white blood cell (WBC) count. Certain aspects of a disease condition may mask abnormalities in a laboratory test. Dehydration may mask anemia or hypoproteinemia. The decrease in plasma volume due to fluid loss will spuriously increase red blood cell (RBC) count and serum protein concentration. Acidosis may mask total body potassium depletion as intracellular potassium ions exchange with extracellular hydrogen ions, resulting in a "normal" serum potassium concentration.

Reference ranges are influenced by many variables, and a number of these will be discussed in the following section. Of particular importance is the individual laboratory performing the tests and the specific methodology employed. For instance, different analyzers and reagent systems used for coagulation tests can greatly influence the results making use of reference ranges from another laboratory inappropriate. It is difficult and potentially misleading to use reference ranges found in the literature for the interpretation of study data. Whenever possible, investigators should establish in-house reference ranges.

Because of a number of limitations, not the least of which is economic, this is not always feasible. For this reason, reference ranges for common hematology and clinical chemistry tests in the eight laboratory animal species described in this book have been included with this chapter (Tables 12.1 through 12.8). These ranges are presented to only serve as general guidelines and to show the differences among the species. They represent a composite of ranges found in the literature and at Covance Laboratories, Inc. When reference ranges are established in-house, it is important that they be periodically updated. Changes in test methodology, animal supplier, and animal husbandry or handling practices are examples of variables that will cause "old" reference ranges to become obsolete.

Reference ranges do not replace the need for control animals in large studies designed to assess the toxic potential of a test material. Because it is not possible to repeatedly duplicate a standard set of study conditions, there is no substitute for age- and sex-matched control animals from the same supplier undergoing the same procedures as the treated animals. For instance, test results from animals receiving a purified diet cannot be compared directly with reference ranges established for animals fed a conventional diet. In small investigational studies with few or no control animals, however, reference ranges may help identify potential toxic effects.

Reference ranges should never be relied upon as the sole means of making judgments concerning the biological importance of a test material–related effect. Values outside of the reference range do not necessarily indicate an abnormal condition, and values within the reference range do not necessarily signify a normal condition. Monkeys tend to have wide reference ranges for many parameters. The finding that the mean serum alanine aminotransferase (ALT) activity of a treated group is significantly higher than that of the control group should not be dismissed as toxicologically unimportant simply because the mean activity of the treated group falls within the upper reference limit. On the other hand, reference ranges for serum urea nitrogen concentration in rats are generally very narrow. If addition of test material to the drinking water of rats caused reduced water consumption, it is quite possible that the mean serum urea nitrogen concentration for these rats will be significantly higher than for the control animals and will exceed the upper reference limit without a meaningful toxic effect on the kidney.

Use of concurrent control animals was recommended by a joint international committee of experts for data interpretation in toxicity studies (Weingand et al., 1996). In addition to concurrent controls and reference ranges, baseline collections (prior to start of dosing) are performed from larger

Table 12.1   Clinical Pathology Reference Ranges for Rats (Young Adult), SD

Test	Units	Male	Female
RBC count	×10⁶/μL	6.7–9.0	5.7–9.0
Hemoglobin	g/dL	13.0–17.0	11.0–17.0
Hematocrit	%	45–54	43–51
MCV	fL	55–65	55–65
MCH	pg	16–22	17–22
MCHC	%	28–34	28–34
Platelet count	×10⁶/μL	700–1500	700–1500
PT	s	12–17	12–18
Partial thromboplastin time	s	17–27	17–27
WBC count	×10³/μL	3.0–14.5	2.0–11.5
Segmented neutrophils	×10³/μL	0.3–3.0	0.01–2.0
Band neutrophils	×10³/μL	0.0–0.0	0.0–0.0
Lymphocytes	×10³/μL	3.0–12.0	1.0–10.0
Monocytes	×10³/μL	0.0–0.5	0.0–0.3
Eosinophils	×10³/μL	0.0–0.3	0.0–0.3
Basophils	×10³/μL	0.0–0.0	0.0–0.0
Nucleated RBC count	/100 WBC	0–2	0–2
Glucose	mg/dL	70–125	70–120
Total protein	g/dL	5.6–7.1	5.5–7.3
Albumin	g/dL	3.9–4.9	4.0–5.2
Globulin	g/dL	1.5–2.3	1.4–2.0
Cholesterol	mg/dL	42–90	45–100
Triglyceride	mg/dL	30–90	15–40
Urea nitrogen	mg/dL	10–16	10–19
Creatinine	mg/dL	0.5–0.8	0.5–0.8
Total bilirubin	mg/dL	0.0–0.2	0.0–0.2
AST	IU/L	60–300	80–250
ALT	IU/L	25–55	25–50
AP	IU/L	85–245	60–110
GGT	IU/L	0–3	0–3
Creatine kinase	IU/L	946–2456	450–2190
Calcium	mg/dL	8.5–10.5	8.5–10.2
Inorganic phosphorus	mg/dL	6.0–9.5	6.0–9.0
Sodium	mmol/L	139–155	139–155
Potassium	mmol/L	4.4–5.7	4.0–5.5
Chloride	mmol/L	100–115	100–113

laboratory animals, such as dog, nonhuman primates, and rabbit, which allow multiple blood collections. These baseline values are important for certain parameters, such as cholesterol, where remarkable individual variability exists between animals, irrespective of their age/sex and source of origin. Collection of baseline clinical pathology samples is not recommended from rodents however, because their test results are usually more uniform and these blood collections have a potential to compromise health of animals prior to start of dosing. Rodent studies typically have larger group size, and prominent age-related changes make the baseline data less useful for comparison with the later time points.

Although limited in conventional toxicology studies, reference ranges do have value. Proper assessment of the significance of laboratory findings is only possible with a firm understanding of

Table 12.2   Clinical Pathology Reference Ranges for Mice (Young Adult), CD-1

Test	Units	Male	Female
RBC count	×10^6/µL	9.0–11.3	9.0–11.5
Hemoglobin	g/dL	13.5–17.0	14.5–17.5
Hematocrit	%	45–55	45–57
MCV	fL	47–55	45–55
MCH	pg	13–16	13–16
MCHC	%	29–34	29–34
Platelet count	×10^3/µL	900–1900	900–1800
PT	s	8–12	10–12
Partial thromboplastin time	s	18–32	18–32
WBC count	×10^3/µL	2.0–10.0	1.0–12.0
Segmented neutrophils	×10^3/µL	0.3–2.0	0.3–2.5
Band neutrophils	×10^3/µL	0.0–0.0	0.0–0.0
Lymphocytes	×10^3/µL	1.0–7.0	1.0–9.0
Monocytes	×10^3/µL	0.0–0.3	0.0–0.3
Eosinophils	×10^3/µL	0.0–0.5	0.0–0.5
Basophils	×10^3/µL	0.0–0.0	0.0–0.0
Nucleated RBC count	/100 WBC	0–2	0–2
Glucose	mg/dL	80–160	80–150
Total protein	g/dL	4.5–7.2	4.2–6.5
Albumin	g/dL	2.8–4.8	2.8–4.5
Globulin	g/dL	1.5–3.0	1.0–2.5
Cholesterol	mg/dL	90–170	50–130
Triglyceride	mg/dL	60–160	40–130
Urea nitrogen	mg/dL	15–45	15–40
Creatinine	mg/dL	0.3–0.8	0.2–0.6
Total bilirubin	mg/dL	0.1–1.0	0.1–0.9
AST	IU/L	70–400	70–400
ALT	IU/L	25–200	25–100
AP	IU/L	30–80	40–100
GGT	IU/L	0–3	0–3
Creatine kinase	IU/L		
Calcium	mg/dL	8.5–11.5	8.5–11.5
Inorganic phosphorus	mg/dL	7.0–11.5	7.0–11.5
Sodium	mmol/L	145–167	145–165
Potassium	mmol/L	5.0–8.5	5.0–8.5
Chloride	mmol/L	110–125	110–125

what is typical for healthy animals under similar conditions. Reference ranges may serve as the starting point, a point of reference, from which one can move toward an appropriate conclusion based on all of the variables that may have impacted a particular study.

SOURCES OF VARIATION IN LABORATORY MEASUREMENTS

The need for developing in-house reference ranges is based on the fact that many variables, such as methodology, affect test results and that controlling these variables is best accomplished within one's own laboratory. In addition, however, there are sources of variation that can affect the results of individual animals within a group and thereby complicate data interpretation.

Table 12.3   Clinical Pathology Reference Ranges for Beagle Dogs (Young Adults)

Test	Units	Male	Female
RBC count	$\times 10^6/\mu L$	5.5–7.7	5.5–7.6
Hemoglobin	g/dL	12.5–17.0	12.3–17.0
Hematocrit	%	38–51	36–50
MCV	fL	60–71	62–72
MCH	pg	20–24	20–24
MCHC	%	32–35	31–35
Platelet count	$\times 10^3/\mu L$	240–550	270–550
PT	s	5.8–8.2	6.0–8.0
Partial thromboplastin time	s	9.0–13.0	9.5–13.5
WBC count	$\times 10^3/\mu L$	6.4–14.6	5.6–15.2
Segmented neutrophils	$\times 10^3/\mu L$	3.0–9.5	3.0–10.5
Band neutrophils	$\times 10^3/\mu L$	0.0–0.0	0.0–0.0
Lymphocytes	$\times 10^3/\mu L$	1.0–6.0	1.5–5.5
Monocytes	$\times 10^3/\mu L$	0.0–0.9	0.0–0.8
Eosinophils	$\times 10^3/\mu L$	0.0–0.8	0.0–0.7
Basophils	$\times 10^3/\mu L$	0.0–0.1	0.0–0.1
Nucleated RBC count	/100 WBC	0–1	0–1
Glucose	mg/dL	82–120	85–125
Total protein	g/dL	5.0–6.5	5.0–6.3
Albumin	g/dL	3.0–3.9	3.1–4.0
Globulin	g/dL	1.6–2.8	1.6–2.8
Cholesterol	mg/dL	120–220	120–230
Triglyceride	mg/dL	16–43	19–45
Urea nitrogen	mg/dL	7–18	7–22
Creatinine	mg/dL	0.5–0.8	0.5–0.8
Total bilirubin	mg/dL	0.0–0.2	0.0–0.2
AST	IU/L	15–45	15–45
ALT	IU/L	22–48	20–50
AP	IU/L	68–190	65–200
GGT	IU/L	0–3	0–3
Creatine kinase	IU/L	35–580	40–540
Calcium	mg/dL	10.2–12.0	9.8–12.1
Inorganic phosphorus	mg/dL	6.6–8.4	6.0–8.2
Sodium	mmol/L	143–158	142–156
Potassium	mmol/L	4.4–6.5	4.3–6.5
Chloride	mmol/L	108–118	109–120

Most variables can be categorized as either physiological, procedural, or artifact. Examples of theses are discussed in the following paragraphs.

Age-Related Changes

Age-related changes must always be considered, and reference ranges should be established in accordance with the age of animals used most often by the individual laboratory. As young animals mature, typical changes in most species include decreasing reticulocyte count, mean corpuscular volume (MCV), serum alkaline phosphatase (AP) activity, serum inorganic phosphorus concentration and increasing RBC count, hematocrit, hemoglobin concentration, total serum protein concentration, and serum globulin concentration. In many species, neutrophil count will increase and

Table 12.4   Clinical Pathology Reference Ranges for Cynomolgus Monkeys (Adult), Wild Caught

Test	Units	Male	Female
RBC count	×10⁶/µL	5.2–7.8	5.5–7.6
Hemoglobin	g/dL	10.5–14.0	10.0–13.5
Hematocrit	%	36–49	34–48
MCV	fL	57–75	57–73
MCH	pg	16–22	16–22
MCHC	%	27–32	27–32
Platelet count	×10³/µL	180–650	175–750
PT	s	9.0–12.5	9.0–12.0
Partial thromboplastin time	s	16.0–29.0	16.0–29.0
WBC count	×10³/µL	5.0–18.0	3.5–18.0
Segmented neutrophils	×10³/µL	0.5–7.5	0.5–9.0
Band neutrophils	×10³/µL	0.0–0.0	0.0–0.0
Lymphocytes	×10³/µL	2.0–12.0	1.0–10.0
Monocytes	×10³/µL	0.0–0.5	0.0–0.5
Eosinophils	×10³/µL	0.0–0.7	0.0–0.7
Basophils	×10³/µL	0.0–0.2	0.0–0.2
Nucleated RBC count	/100 WBC	0–1	0–1
Glucose	mg/dL	43–128	48–135
Total protein	g/dL	7.3–9.2	7.1–9.4
Albumin	g/dL	3.7–5.0	3.5–5.0
Globulin	g/dL	2.9–4.3	2.8–4.5
Cholesterol	mg/dL	110–190	100–200
Triglyceride	mg/dL	20–100	30–80
Urea nitrogen	mg/dL	15–26	12–30
Creatinine	mg/dL	0.8–1.2	0.8–1.3
Total bilirubin	mg/dL	0.1–0.8	0.1–0.8
AST	IU/L	20–100	15–105
ALT	IU/L	15–200	20–230
AP	IU/L	100–1100	150–600
GGT	IU/L	40–170	30–160
Creatine kinase	IU/L	98–1250	88–716
Calcium	mg/dL	8.9–11.3	8.6–11.0
Inorganic phosphorus	mg/dL	5.1–8.9	4.1–8.1
Sodium	mmol/L	139–163	140–166
Potassium	mmol/L	4.0–6.0	4.0–6.5
Chloride	mmol/L	99–112	98–115

lymphocyte count will decrease with age. As a population of animals becomes older, test results exhibit greater variability because of subclinical disease conditions such as progressive nephropathy in rats. Reference ranges therefore become wider, and identification of mild effect becomes more difficult.

Strain and Gender Differences

Strain differences can be important, especially in mice and rats, just as species differences in nonhuman primates. Gender-related differences also occur, but they are often subtle. On the other hand, the effects of estrus may be extremely important, as in ferrets with estrus-induced bone

Table 12.5 Clinical Pathology Reference Ranges for New Zealand White Rabbits (Young Adult[a])

Test	Units	Male	Female
RBC count	×10⁶/µL	5.0–7.2	5.0–7.2
Hemoglobin	g/dL	10.5–15.0	10.5–15.0
Hematocrit	%	32–45	32–45
MCV	fL	55–65	55–70
MCH	pg	19–23	19–23
MCHC	%	30–35	30–35
Platelet count	×10³/µL	300–750	300–750
PT	s	6–9	6–9
Partial thromboplastin time	s	19–23	19–23
WBC count	×10³/µL	4.0–13.0	4.0–13.0
Segmented neutrophils	×10³/µL	1.0–6.0	1.0–6.0
Band neutrophils	×10³/µL	0.0–0.0	0.0–0.0
Lymphocytes	×10³/µL	2.0–9.0	2.0–9.0
Monocytes	×10³/µL	0.0–0.5	0.0–0.5
Eosinophils	×10³/µL	0.0–0.4	0.0–0.4
Basophils	×10³/µL	0.0–1.0	0.0–1.0
Nucleated RBC count	/100 WBC	0–1	0–1
Glucose	mg/dL	105–190	100–190
Total protein	g/dL	5.2–7.5	5.2–7.5
Albumin	g/dL	4.0–5.5	3.5–5.5
Globulin	g/dL	1.0–2.5	1.0–2.5
Cholesterol	mg/dL	25–70	30–100
Triglyceride	mg/dL	50–180	30–180
Urea nitrogen	mg/dL	11–25	11–25
Creatinine	mg/dL	0.9–1.7	0.9–1.7
Total bilirubin	mg/dL	0.1–0.5	0.1–0.5
AST	IU/L	15–45	15–45
ALT	IU/L	15–50	15–50
AP	IU/L	40–140	40–140
GGT	IU/L	0–10	0–10
Creatine kinase	IU/L	150–1000	150–1000
Calcium	mg/dL	13.0–15.5	12.5–15.5
Inorganic phosphorus	mg/dL	3.0–9.0	2.0–9.0
Sodium	mmol/L	133–152	133–150
Potassium	mmol/L	3.5–6.0	3.5–6.0
Chloride	mmol/L	96–106	96–106

[a] Not fasted.

marrow hypoplasia. Axenic animals may have significant differences from others of their species, especially with respect to leukocyte counts and serum globulins.

Effect of Diet

Diet has an effect on laboratory data. Changes in cholesterol are obvious in some species fed atherogenic diets, but less obvious changes can occur in tests such as serum urea nitrogen concentration in animals fed diets containing different types or amounts of protein. Data from animals fed purified diets should always be carefully examined. Small errors in the formulation of these diets can

Table 12.6   Clinical Pathology Reference Ranges for Syrian Hamsters (Adult)

Test	Units	Male	Female
RBC count	×10⁶/μL	6.0–8.5	6.0–8.0
Hemoglobin	g/dL	12.5–16.5	12.0–16.0
Hematocrit	%	35–50	35–45
MCV	fL	52–65	52–65
MCH	pg	19–23	19–23
MCHC	%	32–36	32–36
Platelet count	×10³/μL	300–900	300–900
PT	s	8–15	8–15
Partial thromboplastin time	s	20–24	20–24
WBC count	×10³/μL	4.0–10.0	4.0–10.0
Segmented neutrophils	×10³/μL	0.5–3.5	0.5–3.5
Band neutrophils	×10³/μL	0.0–0.0	0.0–0.0
Lymphocytes	×10³/μL	2.5–8.0	2.5–8.0
Monocytes	×10³/μL	0.0–0.5	0.0–0.5
Eosinophils	×10³/μL	0.0–0.3	0.0–0.3
Basophils	×10³/μL	0.0–0.0	0.0–0.0
Nucleated RBC count	/100 WBC	0–2	0–2
Glucose	mg/dL	50–100	50–100
Total protein	g/dL	5.3–7.0	5.3–7.0
Albumin	g/dL	3.5–4.5	3.5–4.5
Globulin	g/dL	2.0–4.2	2.0–4.2
Cholesterol	mg/dL	50–200	50–200
Triglyceride	mg/dL	152–260	160–264
Urea nitrogen	mg/dL	12–25	12–25
Creatinine	mg/dL	0.3–0.7	0.3–0.7
Total bilirubin	mg/dL	0.1–0.5	0.1–0.5
AST	IU/L	20–100	20–100
ALT	IU/L	20–50	20–50
AP	IU/L	50–200	50–200
GGT	IU/L		
Creatine kinase	IU/L	295–643	336–704
Calcium	mg/dL	10.0–13.5	10.0–13.5
Inorganic phosphorus	mg/dL	4.5–9.0	4.5–9.0
Sodium	mmol/L	145–155	145–155
Potassium	mmol/L	4.0–7.0	4.0–7.0
Chloride	mmol/L	98–110	98–110

have significant effect on health. For most species, fasting is a generally accepted practice designed to help stabilize the test results. However, fasting may actually be a detriment in rabbits and mice.

Excitement and Stress

Excitement and stress can have pronounced effects on laboratory data. Excitement is associated with endogenous catecholamine release and stress with endogenous corticosteroid release. The effects of catecholamines on test results are immediate, whereas those of corticosteroids take longer. The most obvious changes observed in excited or frightened animals (e.g., untrained, unanesthetized monkeys) affect leukocytes and serum glucose concentration. In rats, chronic stress that results in reduced food consumption and/or body weight gain can cause reduction in all hematopoietic cell

Table 12.7   Clinical Pathology Reference Ranges for Guinea Pigs (Adult)

Test	Units	Male	Female
RBC count	×10⁶/µL	4.8–6.8	4.5–6.5
Hemoglobin	g/dL	12.0–16.0	12.0–15.5
Hematocrit	%	38–52	36–50
MCV	fL	70–88	75–90
MCH	pg	24–28	24–28
MCHC	%	28–33	28–33
Platelet count	×10³/µL	300–800	300–900
PT	s	18–26	18–26
Partial thromboplastin time	s	16–28	16–28
WBC count	×10³/µL	2.0–12.0	2.0–12.0
Segmented neutrophils	×10³/µL	0.5–3.5	0.5–4.5
Band neutrophils	×10³/µL	0.0–0.0	0.0–0.0
Lymphocytes	×10³/µL	1.5–10.0	1.5–9.0
Monocytes	×10³/µL	0.0–0.5	0.0–0.5
Eosinophils	×10³/µL	0.0–0.5	0.0–0.5
Basophils	×10³/µL	0.0–0.0	0.0–0.0
Nucleated RBC count	/100 WBC	0–1	0–1
Glucose	mg/dL	75–110	75–110
Total protein	g/dL	5.0–6.3	4.5–6.0
Albumin	g/dL	2.5–4.0	2.2–3.5
Globulin	g/dL	2.5–4.0	2.3–3.8
Cholesterol	mg/dL	25–80	30–80
Triglyceride	mg/dL	10–70	10–70
Urea nitrogen	mg/dL	15–30	15–25
Creatinine	mg/dL	0.5–0.8	0.5–0.8
Total bilirubin	mg/dL	0.0–0.3	0.0–0.3
AST	IU/L	30–80	30–75
ALT	IU/L	30–70	25–65
AP	IU/L	60–100	50–100
GGT	IU/L	5–15	5–15
Creatine kinase	IU/L	200–500	15–500
Calcium	mg/dL	9.5–11.5	9.5–11.5
Inorganic phosphorus	mg/dL	4.0–8.5	4.0–8.5
Sodium	mmol/L	130–142	130–142
Potassium	mmol/L	4.0–6.0	4.0–6.0
Chloride	mmol/L	103–112	103–112

lines (RBCs, WBCs, and platelets; Everds et al., 2013). Excessive handling or restraint for various study procedures or during sample collections has been reported in dogs, monkeys, and other species. In one report, stress-related hematologic changes resolved in monkeys by a week after transportation (Kim et al., 2005). If possible, clinical pathology testing should be delayed for at least a week following shipping to avoid stress-related changes.

Choice of Collection Site and Use of Anesthesia

The most familiar procedural influences on laboratory test results are those associated with sample collection site and the use of anesthesia. Many investigators have analyzed the differences in data resulting from choice of collection site and anesthesia. This is especially true for the rat in

Table 12.8   Clinical Pathology Reference Ranges for Ferrets (Young Adult)

Test	Units	Male	Female
RBC count	×10⁶/μL	7.0–11.0	6.5–10.0
Hemoglobin	g/dL	14.0–18.0	14.0–17.5
Hematocrit	%	45–55	40–50
MCV	fL	50–60	50–60
MCH	pg	18–20	18–20
MCHC	%	32–36	32–36
Platelet count	×10³/μL	300–700	300–900
PT	s	14–17	14–17
Partial thromboplastin time	s	17–20	17–20
WBC count	×10³/μL	4.0–15.0	4.0–15.0
Segmented neutrophils	×10³/μL	2.0–11.0	2.0–11.0
Band neutrophils	×10³/μL	0.0–0.0	0.0–0.0
Lymphocytes	×10³/μL	2.0–7.0	2.0–7.0
Monocytes	×10³/μL	0.0–0.8	0.0–0.8
Eosinophils	×10³/μL	0.0–0.8	0.0–0.8
Basophils	×10³/μL	0.0–0.2	0.0–0.2
Nucleated RBC count	/100 WBC	0–1	0–1
Glucose	mg/dL	90–150	90–150
Total protein	g/dL	5.3–7.5	5.3–7.5
Albumin	g/dL	2.5–4.0	2.5–4.0
Globulin	g/dL		
Cholesterol	mg/dL	100–250	100–250
Triglyceride	mg/dL	13–24	
Urea nitrogen	mg/dL	15–30	15–30
Creatinine	mg/dL	0.3–0.9	0.3–0.9
Total bilirubin	mg/dL	0.1–0.7	0.1–0.7
AST	IU/L	30–120	30–120
ALT	IU/L	50–200	50–200
AP	IU/L	20–100	20–100
GGT	IU/L		
Creatine kinase	IU/L		
Calcium	mg/dL	8.5–10.5	8.5–10.5
Inorganic phosphorus	mg/dL	4.0–8.5	4.0–8.5
Sodium	mmol/L	145–160	145–155
Potassium	mmol/L	4.3–6.0	4.3–6.0
Chloride	mmol/L	110–125	110–125

which variety of bleeding techniques have been used (Mahl et al., 2000; Neptun et al., 1985; Suber and Kodell, 1985). The principle message of these works is that one should use the technique with which they have had good success and with which they are comfortable. While specific study objectives may occasionally dictate which bleeding technique is most appropriate, it is very difficult to consistently obtain high-quality specimens if the techniques are not used routinely. Furthermore, few research facilities have the resources to establish reference ranges for more than one or two bleeding techniques. Choice of bleeding site and anesthesia appear to have their greatest effect on peripheral blood cell counts. Serum enzyme activities, especially for enzymes derived from muscle, can also be affected by bleeding site and anesthesia. Cardiac puncture should not be used if muscle or cardiac markers are of prime interest.

Artifacts

Due to Hemolysis

One of the most common causes of variation is due to artifactual hemolysis. Free hemoglobin may interfere with a variety of assays depending upon the method and instrumentation used. In addition, lysis of erythrocytes releases intracellular constituents such as aspartate aminotransferase, lactate dehydrogenase (LDH), inorganic phosphorus, and potassium that cause spuriously high serum activities and concentrations. There are species differences for erythrocyte intracellular potassium concentration that are discussed later in this chapter.

While hemolysis is generally obvious and can, therefore, be considered when interpreting data, poor laboratory procedures can have effects that are not grossly evident. Intracellular erythrocyte constituents will leach into serum if clotted blood samples are not centrifuged and separated quickly. In addition, serum glucose concentration will decrease at a rate of about 7–10 mg/dL each hour due to erythrocyte metabolism. These changes, along with the potential of analytical drift by laboratory instrumentation, are some of the reasons why randomization of animals for blood collection is extremely important. For example, if control animals in a large study are bled first and high-dose animals last and the serum is not separated from the clotted blood until after the last animal is bled, serum LDH activity and inorganic phosphorus concentration may be statistically lower in the high-dose group simply because their blood did not sit as long before separation and serum glucose lower than control because of erythrocyte metabolism.

Due to Multiple Bleeding Intervals

Sometimes overlooked are the effects of multiple bleeding intervals. It is relatively easy to create iatrogenic, blood loss anemia and hypoproteinemia in animals that are bled frequently for pharmacokinetic studies or for serial test determinations such as plasma cholinesterase activity. In the absence of control animals undergoing the same procedures, it may be difficult to separate test material effects from those of the multiple blood collections.

Various institutions follow guidelines or recommendations from their Institutional Animal Care and Use Committee for maximum allowable blood volumes for various species. These limits for blood volume collection can vary between institutions and depend on several factors such as frequency of collections, species, age/weight, and health/hydration status of animals. In general, the total circulating blood volume is approximately 7% of their body weight in healthy adult animals in most species. According to a guide published by the European Federation of Pharmaceutical Industries Associations and the European Centre for the Validation of Alternative Methods, up to 15% of total circulating volume can be safely removed in a single collection, and up to 20% of total circulating volume can be removed in multiple collections over 24 hours (such as toxicokinetic collections). A single collection of more than 15% of circulating volume is not recommended because that may cause hypovolemic shock if not done slowly (Diehl et al., 2001). However, collecting maximum allowed volume (either a single large volume collection or multiple smaller volume collections) can still impact clinical pathology test results and their interpretation.

Due to Urine Collection Procedures

Time urine collection procedures, as currently practiced in most toxicology laboratories, cannot avoid a number of artifacts in the urinalysis results. It is virtually impossible to make long-term collection (16–24 hours) in such a way as to prevent bacterial contamination and proliferation. Preservatives have disadvantages, and it is very difficult to keep the samples constantly chilled. Because of these

problems, the list of artifacts is long: bacteria proliferate; urine pH increases because urease-producing bacteria result in ammonia formation; highly alkaline urine can cause false-positive proteinuria with reagent strip methods; glucose is consumed by proliferating bacteria; volatile ketones leave solution; bilirubin is oxidized by light to biliverdin, which does not react with reagent strips; cells and casts disintegrate over time; and crystals form, especially those that occur in alkaline urine.

Well-conceived standard operating procedures and careful study design can help to eliminate or reduce many potential sources of variation. However, sources of variation must always be considered when interpreting clinical pathology data.

CLINICAL PATHOLOGY TESTS AND INTERPRETATIONS

Hematology

The hematology tests routinely performed during toxicology studies evaluate erythrocytes, leukocytes, platelets, and coagulation. Many automated cell counters can determine RBC count, hemoglobin, hematocrit, MCV, mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), WBC count, and platelet count from as little as 100 μL of anticoagulated whole blood, but blood collection tubes require large volumes to maintain proper blood-to-anticoagulant ratio. Because erythrocytes of the common laboratory species are smaller than human erythrocytes, species-specific software are used to ensure accuracy. In addition, platelet counts from rodents are often higher than the upper limit for some analyzers (1 million/μL), and adjustments are sometimes necessary for the instrument to report the high counts. Modern hematology analyzers can perform WBC differential counts on animal blood, but microscopic examination of a stained blood film is often necessary to confirm results when abnormalities occur. If an automated cell counter is unavailable, manual assays such as packed cell volume (essentially the same as hematocrit) obtained by microhematocrit tube centrifugation, WBC count performed on a hemocytometer, or estimation of platelet count can still be performed to get reasonable amount of information, but these procedures are very cumbersome and not as accurate or precise as automated hematology analyzers. The common coagulation test, prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen are usually determined by automated coagulation analyzers. Like the cell counters, some of the analyzers must be specifically set up for animal specimens.

In laboratories performing work for safety studies, automated instruments have become a norm because they are faster, more precise, and consistent than manual assays and allow automated data capturing for laboratory information systems.

Erythrocytes

The most common hematology findings in toxicology studies are mild decreases (e.g., <10% from control animal values) in RBC count, hemoglobin concentration, and hematocrit (the percentage of whole blood made up of erythrocytes). The affected animals often exhibit additional findings such as mild reduction in body weight, body weight gain, or food consumption and clinical observations such as dull haircoat, poor grooming habits, and decreased activity. Although specific mechanisms for the erythrocyte effects are typically not identified, there appears to be a generalized reduction of anabolic processes, including erythropoiesis. Considering the normally brisk pace of RBC production (in humans, approximately 100 billion new cells/day), it is not surprising that red cell mass (comprised of RBC count, hemoglobin, and hematocrit) is ultimately affected. Decreased physical activity and correspondingly decreased tissue oxygen demand may also contribute to reduced erythropoiesis. These relatively mild, nonspecific findings for circulating erythrocyte mass are identified most frequently in rat studies where the number of animals/sex/group is usually high

(e.g., 10 animals/sex/group), the dose levels used are typically higher than those for dog or monkey studies, and the normal interanimal variability of hematology data is relatively low. In addition, because the circulating life spans of mouse and rat erythrocytes are shorter than those for dogs and nonhuman primates, similar reductions in erythropoiesis will become apparent for rodents before larger animals. "Anemia" is usually an inappropriate term for these mild effects on RBC count, hemoglobin concentration, and hematocrit and is avoided in regulatory safety studies so as not to imply a toxicologically adverse condition.

Anemia

Anemia is defined clinically as the condition characterized by a hemoglobin concentration below the lower reference limit. RBC count and hematocrit may or may not be proportionately lower, depending on the cause of the anemia and whether or not cell size and hemoglobin content are affected. The erythrocyte indices, MCV and MCHC, measure cell size and hemoglobin concentration. Reticulocyte count and erythrocyte morphology are critical and pieces of information for determining the potential causes of anemia. The general diagnostic approach to anemia is simple. The first step is to determine whether the anemia is regenerative or nonregenerative.

Regenerative Anemia — In regenerative anemia, the hematopoietic tissues are actively trying to replenish the lost erythrocytes by increasing production and release of new erythrocytes into circulation. Following acute blood loss or hemolysis, it takes approximately 3–4 days for the new erythrocytes, or reticulocytes, to appear in peripheral blood. Reticulocytes can be counted manually as a percentage of erythrocytes on a peripheral blood film stained with a vital stain such as new methylene blue. Reticulocyte counts can also be done automatically by some of the newer hematology analyzers. It is best to determine the absolute reticulocyte count by multiplying the reticulocyte count that is no greater than a normal animal. In this case, the animal would not be showing an appropriate regenerative response for its degree of anemia. With the typical Romanowsky stains used for WBC differential counts, reticulocytes are larger and stain slightly more basophilic than the other erythrocytes. During a regenerative response, the erythrocyte morphology would be described by the terms anisocytosis (variable size) and polychromasia (variable color). Nucleated RBCs and Howell–Jolly bodies are often more numerous in peripheral blood films of animals with regenerative anemia, but they also occur in some forms of nonregenerative anemia. Basophilic stippling of erythrocytes is a seldom seen change that may occur during regeneration; it is primarily associated with the lead toxicity, however. During a regenerative response, the erythrocyte indices usually show a higher MCV because of the influx of larger young cells and a lower MCHC because these large cells have a lower hemoglobin concentration. In toxicology studies, increased MCV is more common than decreased MCHC.

The two primary causes of regenerative anemias are blood loss and hemolysis. In addition to the erythrocyte changes, blood loss is usually characterized by lower serum protein concentrations. The source of the blood loss may be identified by clinical observations, necropsy findings, or tests such as fecal and urine occult blood. Hemolytic conditions do not generally cause changes in serum protein concentrations. Hepatosplenomegaly, icterus, bilirubinemia, and bilirubinuria may be associated findings. When hemolysis is suspected, careful examination of the peripheral blood films can sometimes identify the mechanism.

> *Heinz body anemia*: Oxidizing agents may cause Heinz bodies, irreversibly precipitated hemoglobin attached to the internal surface of the erythrocyte membrane. Affected cells are removed from circulation by the mononuclear phagocyte system; the process is called extravascular hemolysis. Although Heinz bodies can usually be seen with the Romanowsky stains, they are more readily identified with vital stains such as those used to count reticulocytes. The size and number of Heinz

bodies observed in chronic low-level exposures may be small and difficult to see. Acute, high-dose exposures cause large, prominent Heinz bodies and a variety of other morphologic changes such as ghost cells (remnants of cell membrane without associated hemoglobin). Agents that produce Heinz bodies have the potential to cause methemoglobinemia and vice versa. Whenever one is observed, the other should be evaluated.

Immune-mediated hemolytic anemia: Many agents can induce immune-mediated hemolytic anemia, although this tends to be an idiosyncratic reaction. There are three general mechanisms by which this occurs: the agent acts as a hapten bound to the erythrocyte membrane, the agent elicits antibody response and the antigen–antibody complex binds to the erythrocyte membrane, or the agent causes the immune system to mistakenly recognize normal erythrocyte antigens as foreign. Immune-mediated hemolysis is also extravascular. Macrophages of the mononuclear phagocyte system may phagocytize entire erythrocytes or just portions of the membrane coated with immunoglobulin. In the latter case, spherocytes are formed that can be identified microscopically on a peripheral blood film. Animals with immune-mediated hemolytic anemia may be severely anemic, but they usually exhibit a pronounced reticulocytosis. The erythrocytes are less osmotically resistant, and the osmotic fragility test has been performed to support the diagnosis. Unfortunately, this test is nonspecific and labor intensive. Antiglobulin or Coombs' tests may help to confirm the diagnosis by identifying immunoglobulin and/or complement on the cell surfaces. This test requires species-specific anti-immunoglobulin or anticomplement. Agglutination indicates a positive test. Occasionally, an animal's fresh whole blood will exhibit autoagglutination in the test tube or on a wet mount of the blood; this is provisional evidence that the animal may have immune-mediated disease. Perhaps the most dependable way to prove that a test material caused immune-mediated hemolysis is to discontinue its administration until the animal exhibits recovery. Rechallenge the animal with the test material.

Hemolytic anemia due to parasites: Hemotropic parasites can cause hemolytic anemia, but with the exception of malaria, caused by *Plasmodium* organisms in nonhuman primates; this is a rare cause of anemia in toxicology studies. Malarial organisms are intracellular and cause hemolysis via multiplication and rupture of the cell. They are readily observed during microscopic examination of blood films (Ameri, 2010), but their appearance in blood fluctuates and it may be necessary to examine blood specimens at multiple intervals. Extracellular organisms that attach to the surface of the cell, such as *Eperythrozoon coccoides* in mice and *Haemobartonella* species in rodents and dogs, are rarely a problem with today's laboratory-reared animals. The infections are generally subclinical, but manifestations of hemolytic anemia can be induced by splenectomy or immunosuppression. Interestingly, subclinical infections in mice may alter certain responses in immune function tests and lead to erroneous conclusions.

Fragmentation anemia: An unusual form of hemolysis that is also rarely observed in toxicology studies is fragmentation anemia. Injury to highly vascular tissue such as the lung, liver, or intestine with the formation of fibrin strands across small vessels can lead to intravascular lysis of erythrocytes as they are "clotheslined" by the fibrin during passage through the vessels. The observation of schistocytes (helmet cells) on peripheral blood films is evidence of the process. Fragmentation anemia may also occur with vascular neoplasms such as hemangiosarcoma. Disseminated intravascular coagulation (DIC) is characterized by, among other things, schistocyte formation. Unfortunately, DIC is usually so severe that the animals bleed to death or die from major organ dysfunction before regeneration is possible.

Nonregenerative Anemia — In nonregenerative anemia, the hematopoietic tissues are unable to respond appropriately to the reduced circulating erythrocyte mass. The anemia is characterized by the absence of polychromasia and reticulocytosis. Although the erythrocytes often appear normal in color (normochromic) and size (normocytic), some types of nonregenerative anemia are distinguished by morphologically distinct cells. The severity of nonregenerative anemias varies according to the etiology.

Anemia of chronic disease and iron deficiency anemia: Chronic inflammatory lesions are associated with mild-to-moderate nonregenerative anemia. This is not unusual in chronic studies. The putative mechanism is a decrease in the transfer of iron to developing erythrocytes. The cells are

normochromic and normocytic. In contrast, iron deficiency, most commonly associated with chronic blood loss or inadequate dietary iron, is characterized by cells that are hypochromic (inadequate hemoglobin) and microcytic. Animals with long-standing iron deficiency anemia have a low MCV and MCHC.

Nonregenerative anemia associated with chronic renal or liver failure: Chronic renal failure is associated with a moderate-to-severe nonregenerative, normochromic, normocytic anemia. Although the mechanism is usually decreased production of erythropoietin, a number of "uremic toxins" have also been implicated in the anemia of chronic renal disease. Chronic liver failure is associated with moderate anemia. Acanthocytosis, an erythrocyte morphologic abnormality characterized by several blunt cytoplasmic projections resembling pseudopodia, is sometimes a feature of this anemia. The mechanism is believed to be an imbalance in the cholesterol/phospholipid ratio in the cell membrane. As with anemia of renal failure, the anemia of liver failure is not nearly as significant to the animal as the primary organ dysfunction. Endocrine disorders such as hypothyroidism and hypoadrenocorticism may also have mild nonregenerative anemia as part of the disease syndrome.

Nonregenerative anemia associated with leukemia: Nonregenerative anemia is typically a feature of leukemia. The cells are generally normochromic and normocytic unless it is the erythrocytic line that is neoplastic. The principle mechanisms of the anemia are the "crowding out" of normal hematopoietic tissue by the neoplastic cell proliferation and competition for nutrients. An interesting exception is large granular lymphocyte leukemia of Fischer-344 rats. This form of leukemia is associated with a predictable immune-mediated hemolytic anemia that is regenerative.

Aplastic anemia: This rare form of anemia occurs when hematopoietic pluripotent stem cells are injured, as in irradiation or benzene toxicity. The anemia becomes progressively worse because senescent cells are not replaced. Typically, however, the animal will die from the consequences of severely decreased WBC count (infections) or platelet count (hemorrhage) long before it becomes severely anemic. This is because the circulating survival time of erythrocytes is several weeks, whereas it is only about a week for platelets and about a day for most leukocytes. Agents that damage the microenvironment necessary for hematopoiesis can have similar effect. In addition, it is possible for a single cell line to be severely depleted without an effect on the others.

Megaloblastic anemia: Humans that have folate or vitamin B_{12} deficiency have macrocytic erythrocytes because these nutrients are necessary for deoxyribonucleic acid (DNA) synthesis and developing erythrocytes undergo fewer divisions before maturation. This form of anemia is also uncommon and referred to as megaloblastic because asynchronous development of erythrocyte precursors is observed in the bone marrow smears. Although folate and vitamin B_{12} deficiency are not a problem in animals, agents such as methotrexate that block folate synthesis, or cyclophosphamide, an alkylating agent that inhibits DNA synthesis, can cause megaloblastic anemia in animals.

Polycythemia

Increased RBC count, polycythemia, is fairly unusual in toxicology studies. The most common cause of relative polycythemia is simple dehydration. Relative polycythemia may also be observed secondary to lung disease that causes systemic hypoxia and triggers erythropoietin production. A similar mechanism occurs with systemic alkalosis. By increasing the affinity of hemoglobin for oxygen, alkalosis causes the renal tissue sensors to detect hypoxia, triggering erythropoietin production. In addition to these conditions, drugs that induce erythrocyte production (erythropoietin or erythropoietin-stimulating agents) can produce polycythemia. However, features of "iron deficiency" may be evident in erythrocyte indices when iron stores are depleted after a period of sustained reticulocytosis.

Leukocytes

The examination of leukocytes is part of the minimum hematologic database. It includes the quantitative determination of total and differential WBC counts and the qualitative assessment of

cellular morphologic abnormalities. The differential WBC count enumerates granulocytes (neutrophils, eosinophils, and basophils), lymphocytes, and monocytes. Increased numbers of these cells are called neutrophilia, eosinophilia, basophilia, lymphocytosis, and monocytosis, respectively. Neutropenia, eosinopenia, lymphopenia, and monocytopenia refer to decreases. In addition to these leukocytes, some newer hematology analyzers (ADVIA) also classify a subset of leukocytes as large unstained cells (LUCs). These cells are in very small proportion and represent atypical large lymphocytes or monocytic cells that may increase with inflammatory response or decrease with lymphopenia or marked generalized decrease in leukocytes. The normal cell counts for basophils and LUCs are so low that decreases are difficult to recognize.

Relative counts (percentages) for the different types of leukocytes, obtained by doing the differential count, are of little or no value without knowledge of the total WBC count. A dog with 70% neutrophils could be normal or have neutrophilia or neutropenia depending on the total WBC count. By looking only at the relative count data, a 90% lymphocyte count with 100,000 lymphocytes/μL cannot be distinguished from a 90% lymphocyte count with 5,000 lymphocytes/μL. Because it is not always possible to microscopically distinguish neoplastic lymphocytes from normal lymphocytes, nothing in the reported data would indicate the possibility of lymphocytic leukemia in the first case. In light of these discussions, only absolute differential WBC counts should be used for interpretation.

Neutrophils and lymphocytes are the principle cell types found in peripheral blood, and toxicologic effect on leukocytes usually involves one or both of these cell lines. While primary effects occur, the changes observed are most commonly secondary changes in response to primary toxicity of other tissues or organ systems.

Excited or frightened animals may have a physiological leukocytosis (increased WBC count) due to endogenous catecholamine release. Increased heart rate, blood pressure, or muscular activity mobilizes cells that normally marginate along the endothelium of smaller vessels. The addition of these cells may more than double the number flowing freely in circulation and consequently increase the WBC count proportionately. Neutrophilia and lymphocytosis can both occur.

A steroid or stress-induced leukocyte response, "stress leukogram," refers to a combination of changes observed in animals receiving corticosteroids or producing increased endogenous corticosteroids because of some stressful condition. It generally consists of a mature neutrophilia (no immature neutrophils such as bands or metamyelocytes), lymphopenia, and sometimes monocytosis depending on the animal species. The mature neutrophilia develops as a consequence of increased release of segmented cells from the bone marrow storage pool, decreased margination of cells, decreased movement of cells into tissues, and increased stability of lysosomal membranes. Lymphopenia results from steroid-induced lysis and cell redistribution. Eosinopenia develops as a result of decreased production and release from the bone marrow. And monocytosis, when it occurs, is thought to result from mobilization of marginated cells. It is interesting that a classic "stress leukogram" is a relatively infrequent finding in toxicology studies even though the study design or the test material often creates physical conditions that appear to be quite stressful.

Neutrophils

The primary function of the neutrophil is phagocytosis of small particulate matter (e.g., bacteria). The neutrophil is also an integral cellular component of inflammation. It is therefore not unusual to observe neutrophilia secondary to nearly any inflammatory lesion caused by a test material. The term "left shift" indicates an increased number of immature neutrophils in circulation. A left shift can occur whenever an inflammatory lesion has a significant demand for neutrophils and immature cells are released from the bone marrow. Lesions that cause a left shift are almost always easily

identified, if not by physical examination and the evaluation of other laboratory data, then certainly at necropsy. They frequently involve infectious organisms that have invaded tissue damaged by the test material. A "degenerative" left shift describes the situation of a normal or decreased neutrophil count with more immature than mature neutrophils. It generally indicates a severe infection such as might occur with aspiration pneumonia or during a study using indwelling intravenous catheters that become contaminated, leading to systemic bacterial infection. In severe conditions such as these, when the demand for neutrophils is extreme, so-called toxic neutrophils may be observed. These are neutrophils with morphologic changes such as cytoplasmic basophilia, vacuolation, or granulation and Dohle bodies (small, bluish-gray cytoplasmic inclusions that represent aggregated rough endoplasmic reticulum).

Neutropenia occurs for three primary reasons. Decreased production of neutrophils is frequently observed in safety studies with cytotoxic drugs (chemotherapeutic drugs, inorganic solvents, and estrogens) or irradiation that causes bone marrow suppression/toxicity. There may be extreme demand for and consumption of neutrophils as described earlier. There may be sequestration of neutrophils along the endothelium in capillary beds as occurs in endotoxic shock. Chemotherapeutic drugs that cause damage to myeloid precursors also affect erythrocyte and platelet production, but neutropenia and increased susceptibility to bacterial infection will be the hematologic problem first encountered. On rare occasion, drugs may induce immune-mediated neutropenia.

Lymphocytes

These cells are responsible for a wide variety of immune system functions. Although there are many lymphocyte subpopulations, it is not possible to distinguish them by light microscopic examination. Lymphocytes are unique among leukocytes in that they recirculate. That is, lymphocytes leave the vascular system through venules in lymph nodes and ultimately return to the blood through the thoracic duct. They are long-lived cells compared with other leukocytes. The most common cause of lymphocytosis is the physiological lymphocytosis associated with excitement, as previously described. Occasionally, increased lymphocyte counts are observed with chronic infections (especially in rodents) and much less frequently with hypersensitivity syndromes and immune-mediated diseases.

Much more common than lymphocytosis is the presence of morphologically distinct lymphocytes sometimes called "reactive" lymphocytes or immunocytes. They can be seen in low numbers secondary to any type of antigenic stimulation and are so common that no mention is usually made of their presence.

Lymphopenia occurs most frequently as a part of the steroid or stress-induced leukocyte response. Agents that cause neutropenia, such as chemotherapeutic agents, will usually cause lymphopenia as well. Because of the many subpopulations of lymphocytes, it is difficult to know the biological significance of a small change in lymphocyte count. It is possible that a small change could represent an effect on a specific subpopulation of cells. This is the case with HIV infection in humans.

Eosinophils, Basophils, and Monocytes

Absolute eosinophil, basophil, and monocyte counts are normally very low (usually <1000/μL) and quite variable. It is very unusual therefore to be able to detect toxicologic effects on these cell types. Eosinophilia may occur secondary to some hypersensitivity syndromes. Eosinopenia may result from the steroid or stress-induced leukocyte response. The primary function of the monocyte is phagocytosis and digestion of large particulate matter such as senescent cells, necrotic cellular debris, and large microorganisms. Monocytes process antigens and present them to lymphocytes

in a more antigenic form. Monocytosis may occur secondary to lesions involving extensive tissue destruction such as neoplasms with associated necrosis or hemolytic anemia.

Leukemia

It is not unusual for small percentage of rodents (control and treated animals) to develop leukemia in a carcinogenicity study. Animals with leukemia do not always have elevated WBC counts, and neoplastic cells may be difficult to find on peripheral blood films (referred as "aleukemic leukemia"). Unfortunately, when neoplastic cells are present in large numbers, it is often difficult to identify the specific cell type using routine staining procedures and light microscopic examination. Because of these factors, diagnosis of leukemia is more easily and reliably made by the histopathologic examination of study animals than by periodic examination of the blood as required by regulatory guidelines.

Platelets

Almost immediately following vascular injury, platelets adhere to exposed collagen and begin to aggregate, forming a primary platelet plug that is sufficient to control bleeding from minor injuries of very small vessels. The aggregated platelets release a variety of substances that stimulate vasoconstriction and fibrin formation. The fibrin acts to cement the mass of platelets into a stable hemostatic plug. Signs of thrombocytopenia (decreased platelet count) include petechial and ecchymotic hemorrhages (most easily observed in mucous membranes), melena, and prolonged bleeding from small wounds such as venipuncture sites. Signs are generally not apparent until the count is less than 50,000/μL. Platelet function defects, such as that caused by aspirin, may cause identical signs, but the tendency to do so is much less. In addition to platelet count, platelet function studies measuring adhesion and aggregation are available but impractical as standard tests. Bleeding time is an in vivo test that measures the functional ability of platelets to stop the bleeding from a controlled superficial wound. Although commonly used in human medicine, this test is difficult to standardize in animals. It should be reserved for investigational purposes.

Thrombocytopenia

This occurs as a result of either decreased production or increased consumption of platelets. Toxins that affect erythroid and myeloid progenitor cells often cause injury to megakaryocytes (platelet precursors). Because the circulating life span of platelets is about 7–10 days, acute toxicity of megakaryocytes leads to decreased platelet count in about a week. Increased consumption of platelets may be caused by immune-mediated phenomena or DIC. In the latter, prolonged coagulation times, decreased plasma fibrinogen concentration, and increased fibrin/fibrinogen degradation products (FDPs) are associated findings. One clue for differentiating decreased production from increased consumption is the presence of large macroplatelets in peripheral blood. These generally indicate increased production of young platelets that are larger than normal.

Thrombocytosis

Increased platelet count, thrombocytosis, is rarely a primary effect of a test material. It may be observed as a secondary effect in conjunction with generalized bone marrow stimulation as in hemolytic anemia or some inflammatory diseases. Thrombocytosis is also associated with iron deficiency. The increase in platelet numbers that occurs in toxicology studies is generally small and not likely to have any biological significance. If platelet counts are markedly elevated, however, the potential for thromboembolic events is increased.

Coagulation

The coagulation mechanism is traditionally divided into two pathways. The intrinsic pathway, routinely evaluated by the APTT or the activated coagulation time, begins with exposure of factor XII to subendothelial collagen or other abnormal surfaces. The extrinsic pathway, evaluated by the one-stage PT, is initiated by exposure of facto VII to tissue thromboplastin. Both mechanisms share the terminal sequence of events, including conversion of prothrombin to thrombin, which in turn converts fibrinogen to fibrin. For routine coagulation tests (PT, APTT, and fibrinogen), citrated plasma is used and blood collection tubes are designed to achieve a 9:1 ratio for blood/sodium citrate. This blood-to-anticoagulant ratio is critical, and alteration in blood volume (short filling the tubes) will significantly impact the results rendering the results incomparable to the reference range or with the control animals.

The assays are not particularly sensitive to small changes in the concentration of clotting factors. In general, the activity of a single factor must be reduced below approximately 30% of normal before coagulation times are prolonged. Given the conditions in most toxicology studies, where animals are exposed to the test material for a prolonged period of time, if there were a significant effect on the production of a clotting factor, it is likely that the animals would exhibit some form of bleeding diathesis. These include severe hemorrhage externally (e.g., epistaxis or hematochezia) or internally (e.g., subcutaneous hematoma, hemothorax, or hemarthrosis).

The majority of clotting factors are synthesized by the liver. Liver injury and dysfunction may cause depletion of clotting factors sufficient to prolong the coagulation assays. The liver requires vitamin K for the production of functional forms of factors II, VII, IX, and X. Vitamin K antagonists, such as warfarin and diphacinone, cause prolongation of both the intrinsic and extrinsic mechanism assays. Because factor VII has the shortest half-life and is part of the extrinsic mechanism, prolonged PT occurs before prolonged APTT. Since vitamin K is a fat-soluble vitamin, coagulation assays are indicated when test materials are administered that have the potential of depleting fat-soluble vitamins. This is true of synthetic fats that are not absorbed by the intestine.

Depletion of all clotting factors with subsequently prolonged coagulation times is a feature of DIC. Fibrinogen concentrations and FDP assays are used to help confirm the diagnosis.

Small, statistically significant differences in mean PT and APTT between control and treated animals (e.g., 2–3 s) are occasionally observed in toxicology studies. This degree of change has little or no biological impact on the individual animals. However, it should not be casually dismissed because it may be an early indication of a potential problem. Depending on the test material, it may be necessary to design longer studies or increase the dose levels to see if the effect is repeatable and meaningful.

It is imperative that reference ranges, if used, should be from the same lab and with current instrument and reagents. Due to the nature of coagulation cascade, different instruments or change of a reagent on the same instrument can produce remarkably different results, and various species may respond differently to a reagent change. Also certain excipients or preservatives used in test material formulations can impact coagulation results, e.g., polyvinyl pyrrolidone and polyethylene glycol have been reported to cause APTT prolongation on certain instrument/reagent systems (Bakaltcheva et al., 2000).

Clinical Chemistry

The clinical chemistry tests routinely performed during toxicology studies generate information concerning carbohydrate, lipid, and protein metabolism, renal function, liver function, hepatocyte injury, and electrolyte balance. Advances in clinical chemistry instrumentation have greatly reduced sample volume requirements and have therefore enabled the laboratory to produce

complete biochemical profiles on animals as small as rats without compromising the study because of excessive blood collection. Most instruments can use 250 μL of serum or less to run a 17–18 test panel. The vast majority of common tests do not require modification of the methods used for testing human samples. There are, however, many differences in the expected ranges of results for individual species. When purchasing an instrument and reagents for work with animal specimens, volume requirements (including dead space) and ranges of linearity are important considerations.

Carbohydrate, Lipids, and Proteins

Glucose

Serum glucose concentration depends upon intestinal absorption, hepatic production, and tissue uptake of glucose. The balance between hepatic production and tissue uptake is influenced by a variety of hormones including insulin, glucagons, corticosteroids, adrenocorticotropic hormone (ACTH), growth hormone, and catecholamines. Insulin is the primary factor responsible for the uptake of glucose by tissues. Corticosteroids, catecholamines, and growth hormone are called insulin antagonists because they interfere with insulin's action on cells. Furthermore, glucagons and glucocorticoids stimulate hepatic gluconeogenesis, and glucagons and catecholamines glycogenolysis. These actions tend to increase serum glucose concentration. The practice of fasting animals prior to blood collection decreases the variability that accompanies postprandial intestinal absorption of glucose. Another procedural consideration for glucose analysis is prompt separation of the serum from clotted blood. As mentioned previously, erythrocyte, and to a lesser degree leukocyte, glycolysis will reduce serum glucose concentration by approximately 7–10 mg/dL every hour that the blood cells remain in contact with the serum at room temperature.

Hyperglycemia and Hypoglycemia — The most frequently encountered causes of hyperglycemia are failure to fast an animal and catecholamine release secondary to excitement or fear. Animals that become moribund occasionally develop hyperglycemia. Less frequently encountered causes, especially in toxicology studies, include insufficient insulin (diabetes mellitus and pancreatitis) and increased glucocorticoids (hyperadrenocorticism and steroid therapy). Hypoglycemia may result from improper handling of the specimen, malnutrition, malabsorption, severe hepatic disease, endotoxemia, and some tumors, in particular, insulinomas and hepatomas. Occasionally, in toxicology studies, treated animals that fail to thrive and gain body weight will also have mildly lower serum glucose concentration (e.g., 10–15 mg/dL) than the control animals even though there are no differences in food consumption. Although the mechanism for this phenomenon is not clear, two possibilities are poor assimilation of the food and alteration of the body's "set point" for serum glucose. Regardless of the cause, the reduction is probably of little biological importance and is simply a reflection of the overall process that has caused the animals to do poorly.

Cholesterol and Triglycerides

Cholesterol is required for the biosynthesis of bile acids, corticosteroids, and sex steroids. Triglycerides serve as an important source of energy. Serum cholesterol and triglycerides are derived from dietary intake and endogenous synthesis, primarily by the liver. The liver, via the biliary system, is the major excretory pathway for cholesterol. In circulation, cholesterol and triglycerides are components of chylomicrons and the lipoproteins: very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL). Chylomicrons, which cause serum to appear lipemic, are produced by intestinal cells after a fatty meal and are rich in triglycerides. Hepatocytes synthesize VLDL, which has less triglyceride than chylomicrons but more cholesterol. The triglycerides in chylomicrons and VLDLs are broken down to free fatty acids and

monoglycerides by lipoprotein lipase attached to the surface of endothelial cells, especially in the capillaries of adipose tissue and muscle. Adipocytes tend to reesterify the fatty acids for storage as triglycerides. Muscles tend to oxidize the fatty acids for energy. The loss of triglyceride causes VLDL to become LDL. In humans, about two-thirds of serum cholesterol is transported by LDL. In contrast, HDL, generated by the liver and other tissues, is the principal lipoprotein responsible for cholesterol transport in several animal species. Species differences in lipid metabolism make it quite difficult to correlate effects in animal models with those in humans. For example, rats normally have very low LDL cholesterol, and rabbits are extremely sensitive to atherogenic diets where marked increases occur in cholesterol, primarily due to increased VLDL (Campbell, 2004).

Serum triglyceride concentration is elevated postprandially, while serum cholesterol concentration is relatively stable. Both are elevated in hypothyroidism and diabetes mellitus. Cholesterol is the predominant lipid in hypothyroidism, and triglycerides predominate in diabetes mellitus. In both cases, lipoprotein lipase activity is reduced.

Biliary stasis, whether intrahepatic or extrahepatic, and other forms of liver disease can increase serum cholesterol concentration, but severe liver disease causing hepatic dysfunction may be associated with hypocholesterolemia.

Nephrotic syndrome, which is almost always the result of glomerular injury, is characterized by increased urinary protein excretion, hypoalbuminemia, and hypercholesterolemia. Steroid therapy, and perhaps hyperadrenocorticism, is also associated with increased serum cholesterol concentration. As might be expected, nutrient deficiency and malassimilation can cause decreased serum cholesterol concentration.

Effects on serum cholesterol concentration are relatively frequent findings in toxicology studies. Both increases and decreases are observed. While the changes are usually small and generally believed to represent minor alterations in lipid metabolism, the exact mechanisms involved are rarely identified. Many factors are probably involved, including food consumption and assimilation, body weight and composition, activity, and hormone balance.

Protein

Total serum protein concentration is a measure of all of the different proteins in plasma with the exception of those that are consumed in clot formation such as fibrinogen and the clotting factors. For this reason, plasma protein concentration is generally about 0.3–0.5 g/dL higher than serum protein concentration.

Albumin: Albumin is the most abundant individual protein and is largely responsible for maintaining intravascular oncotic pressure. Albumin also serves as a storage reservoir of amino acids and as a transport protein, binding most plasma constituents that do not have a specific transport protein.

Globulins: The globulins constitute a heterogeneous population of proteins, including specific transport proteins (e.g., transferrin for iron, lipoproteins for lipids, haptoglobin for hemoglobin, and thyroxine-binding globulin for thyroxine), mediators of inflammation (e.g., complement and C-reactive protein), clotting factors (e.g., fibrinogen, thrombin, and factor VIII), catalysts and inhibitors of biochemical reactions (e.g., enzymes), and immunoglobulins (e.g., IgG, IgM, and IgA). Globulins are nonspecifically categorized by their electrophoretic migrations pattern as α-, ß-, and γ-globulins. Depending on the animal species, these regions may be further subdivided; most species have two α-regions: alpha$_1$ and alpha$_2$. Immunoglobulins are generally found in the γ-regions of the electrophoretogram, but some, IgM in particular, also extend into the ß-regions. The liver synthesizes albumin and most of the globulins, with the major exception of immunoglobulins.

Serum protein electrophoresis: In most laboratories, total serum protein and albumin concentrations are measured directly, and serum globulin concentration is calculated by subtraction. Serum protein electrophoresis is sometimes valuable for determining the cause of increased or decreased globulin concentration, and its use should be reserved for that purpose. A broad-based increase

in γ-globulins, generally the results of antigenic stimulation, is termed a polyclonal gammopathy because plasma cells have produced a heterogeneous population of immunoglobulins in response to the antigenic challenge. A narrow-based increase in γ-globulins is termed a monoclonal gammopathy and is usually the result of a single immunoglobulin class being produced in excess by a single clone of lymphocytes or plasma cells. Most frequently, but not always, the clone of cells is neoplastic (e.g., multiple myeloma). A broad-based increase of the α-globulin regions, especially alpha$_2$, frequently occurs secondary to inflammation and the production of acute-phase reactant proteins. These acute-phase proteins (e.g., alpha$_2$-macroglobulin, haptoglobin, and ceruloplasmin) are part of the general response to inflammation.

The cost/benefit ratio of serum protein electrophoresis is high, and it should not be routinely run as part of the minimum database. Unfortunately, some regulatory guidelines recommend routine serum protein electrophoresis in safety assessment studies. If the production of a single, specific protein in α- or ß-regions is affected by a test material, the absence of change in the other proteins of the same region will probably be sufficient to mask any effect on the electrophoretogram. On the other hand, if the test material does affect a particular region, further identification procedures are necessary because electrophoresis does not identify specific globulin proteins.

Hyperproteinemia: The most frequent causes of hyperproteinemia are dehydration and polyclonal gammopathy secondary to antigenic stimulation. Monoclonal gammopathies are rare in toxicology studies. Dehydration doesn't cause true hyperproteinemia, but a relative increase in proteins (due to reduction in fluid component of blood) and serum albumin and globulin concentrations are increased proportionately.

Hypoproteinemia: This results from either decreased production or increased loss of protein. In dietary toxicity studies, decreased protein production may result from effects on food consumption, digestion, or absorption. Because of the reserve capacity of the liver, hepatic injury must be fairly severe before protein synthesis is notably diminished. However, in large studies, small differences between the control and treated groups may be apparent with mild-to-moderate hepatotoxicity. Loss of protein, both albumin and globulin, occurs with hemorrhage and exudative lesions such as burns. Albumin is the principle protein lost as a result of enteropathies and glomerulopathies. The half-life of albumin is shorter in smaller species: approximately 2 days for mice and approximately 8 days for dogs. Theoretically, impaired albumin synthesis or albumin loss can be detected earlier in the rodents. Hydration status of the animal is always an important factor for proper interpretation of changes in serum protein concentrations. Hypoproteinemia, like anemia, can be masked by dehydration. A small, often statistically significant decrease in serum albumin concentration is one of the most frequent findings in toxicology studies. The exact mechanism is usually not apparent but a combination of factors, similar to those causing mildly lower glucose, is probably responsible.

Renal Function

Serum urea nitrogen concentration and serum creatinine concentration are used in conjunction with urine specific gravity or osmolality to evaluate renal function. These tests are relatively insensitive to small effects on the kidney and a number of nonrenal causes for serum elevations that must be considered.

Urea Nitrogen

Urea is synthesized by the liver from ammonia that is absorbed from the intestine or generated by endogenous protein catabolism. It is freely filtered through the glomerulus and excreted in urine. Some urea is passively reabsorbed with water in the proximal tubule; the amount that is reabsorbed is inversely related to the rate of urine flow through the tubules. Decreased glomerular filtration

rate (GFR) causes serum urea nitrogen concentration to increase. However, because urea production can vary with diet or protein catabolism, increased serum urea nitrogen concentration, termed azotemia, can be categorized as prerenal, renal, or postrenal.

Prerenal Azotemia — This type of azotemia develops as a result of increased hepatic urea synthesis or decreased renal blood flow. The former may result from high-protein diets or conditions that increase protein catabolism such as starvation, fever, infection, tissue necrosis, and high gastrointestinal hemorrhage. Decreased renal blood flow, with subsequently decreased GFR, may result from dehydration, shock (hemorrhagic or circulatory), or cardiovascular disease. The changes in serum urea nitrogen concentration caused by increased urea synthesis are typically small, but those caused by decreased renal perfusion are dependent on the degree of GFR reduction and can be quite large. The concentrating ability of the kidney is not affected by these conditions. In the case of prerenal azotemia due to dehydration, the kidneys attempt to conserve body water and urine specific gravity is elevated.

Renal Azotemia — This type of azotemia develops as a result of primary renal disease or toxicity. The renal lesions can be acute or chronic. Conventionally, serum urea nitrogen concentration is considered insensitive indicator of renal function and believed to remain unaffected until approximately 75% of the kidneys' nephrons are damaged and nonfunctional. However, that concept is probably more applicable to clinical settings where results from a single animal are being compared with the reference range for determination of azotemia. In toxicology studies, changes in urea nitrogen are usually identified at an earlier stage because of larger group size, comparison with concurrent control, and correlation with clinical signs and histopathology findings.

By the time renal failure is detected, renal concentrating ability is usually impaired and urine specific gravity is isosthenuric (i.e., the same as the glomerular filtrate, 1.008–1.012). An indication of the chronicity of the renal lesions may be gleaned from the hematology data. Concurrent nonregenerative anemia suggests that the lesions are chronic.

Postrenal Azotemia — This type of azotemia results from obstruction of the urinary outflow tract. This is rarely observed in toxicology studies, but test materials that promote urinary calculi formation might cause this condition.

Creatinine

This is a nonprotein nitrogenous waste material that is freely filtered by the glomerulus and, unlike urea, is not reabsorbed by the tubules. It is formed at a fairly constant rate by the breakdown of creatine, a molecule that stores energy in muscle as phosphocreatine. Serum creatinine concentration is influenced by muscle mass and conditioning but is relatively independent of dietary influences and protein catabolism. Although it tends to rise and fall more slowly, serum creatinine concentration parallels changes in serum urea nitrogen concentration caused by alterations in renal blood flow, renal function, or urinary outflow.

Endogenous creatinine clearance is sometimes used as a measure of GFR because blood levels of creatinine are relatively stable over short intervals. Creatinine is freely filtered and not significantly secreted or reabsorbed. Mice are an exception where secretion from renal tubular cells accounts for up to 50% of total excreted creatinine and endogenous creatinine clearance is not a reliable measure of GFR (Eisner et al., 2010).

Endogenous creatinine clearance may underestimate the true GFR because of the presence of noncreatinine chromogens that spuriously increase the measure of serum creatinine concentration but not urinary creatinine concentration.

Liver Function and Hepatocyte Injury

The critical metabolic, synthetic, and excretory roles of the liver and the abundant enzymatic machinery needed to perform these functions result in a large number of biochemical parameters that may be altered due to toxicity. On the other hand, the large functional reserve of the liver makes possible a significant loss of tissue with minimal or no detectable change in routine laboratory tests. While no single test is superior for detecting liver toxicity, the pattern of abnormal findings in a battery of tests may help to determine the location and severity of liver lesions.

Many enzymes have been identified that have increased serum activity when hepatocellular damage is present. Although these enzyme activities are not measure of liver function, they can detect cellular degeneration or necrosis. The utility of a particular enzyme depends on a number of factors, including relative specificity to liver, intrahepatic location, intracellular location, concentration gradient between cell and serum, serum half-life, in vitro stability, and the ease, accuracy, and economy of measurement. ALT, aspartate aminotransferase (AST), sorbitol dehydrogenase (SDH), LDH, and glutamate dehydrogenase (GDH) are some of the enzymes that have been used to evaluate hepatocellular damage. Each has advantages and disadvantages, some of which depend on the laboratory animal species being tested.

Serum ALT Activity

In general, serum ALT activity, formerly known as serum glutamic pyruvic transaminase, is the most useful enzyme activity for identifying the presence of hepatocellular damage. The enzyme is found in many tissues, but its greatest concentration in most species is within hepatocytes. For practical purposes and in the absence of severe muscle necrosis, significant elevations of serum activity result only from hepatocyte ALT. The enzyme is primarily cytosolic, and its concentration within the cell is up to 10,000 times greater than that in the serum. Therefore, ALT may "leak" into serum in any condition that alters membrane permeability to a sufficient degree. This does not require cell death; elevated activity does not imply necrosis. The magnitude of serum activity elevation is proportional to the number of affected hepatocytes and is not indicative of the reversibility of the lesion. For example, it is possible to have higher serum ALT activity following reversible cellular hypoxia secondary to hypovolemic shock than might occur with focal necrosis caused by localized hepatic abscess. Of course, the greatest elevations result from severe lesions that affect a large portion of the liver tissue.

Following an acute hepatotoxic episode, serum ALT activity can rise within 1 hour. After a single dose or exposure to toxicant, the activity will peak in 1–2 days and then decline. So if clinical pathology tests are run 1 or 2 weeks after a single administration of the test material, the elevations in ALT activity may be missed even if the test material causes marked hepatotoxicity (e.g., carbon tetrachloride). Prolonged elevations may reflect increased production of ALT in regenerative liver tissue or continued "leakage" from cells whose close proximity to the primary lesion has resulted in degenerative changes secondary to the altered microenvironment.

Increased serum ALT activity is not specific for primary hepatocellular disease. Increased ALT can occur with bile duct obstruction where accumulation of bile salts can physically damage the cell membranes of surrounding hepatocytes and also with extrahepatic factors such as muscle injury or necrosis (Boone et al., 2005; Ennulat et al., 2010b). Drugs such as corticosteroids and anticonvulsants appear to induce ALT production. Because these drugs can also cause pathological changes that result in high serum enzyme activity, it is difficult to determine whether an elevation is due to enzyme induction or drug-induced disease without histopathologic examination (Müller et al., 2000).

Serum AST and LDH Activities

Serum AST (formerly serum glutamic oxaloacetic transaminase) and LDH activities tend to parallel serum ALT activity with respect to liver damage. Unfortunately, they are not liver specific because of high concentrations in other tissues, especially muscle. In small species, blood collection techniques sometimes cause increased variability, reflected in wider reference ranges. There appears to be no advantage in determining both of these enzyme activities, and AST is generally preferred. In most species, elevations in serum AST activity due to hepatotoxicity are not as pronounce as the elevations in serum ALT activity; this may occur because a portion of AST is mitochondrial. Corticosteroids and anticonvulsants affect AST in a similar manner as ALT. Decreased serum activities of AST and/or ALT are occasionally observed in toxicology studies and may indicate decreased hepatocellular production or release, inhibition of the enzymes' activity, interference with the enzyme assay, or an effect on the coenzyme, pyridoxal 5′-phosphate (vitamin B_6). Decreased serum activity of these enzymes has not been shown to be a pathologically important phenomenon.

Serum SDH and GDH Activities

Serum SDH and GDH activities, neither of which are used in human medicine in the United States, have occasionally been recommended as good indicators of hepatic toxicity in laboratory animal species. Increased serum activity of each enzyme is liver specific; SDH is a cytosolic enzyme and GDH is mitochondrial. Theoretically, in order for serum GDH activity to increase, the cell damage must be relatively severe. Because of the higher GDH activity in centrilobular region than in the periportal area, GDH elevations are more pronounced in conditions involving centrilobular necrosis (O'Brien et al., 2002). Elevations in serum SDH activity return to baseline levels faster than for other liver enzymes because of a short serum half-life. The major drawback for both of these enzymes is the assay. Their lack of popularity in human medicine is responsible for the absence of standard automated procedures.

Another enzyme, alpha-glutathione *S*-transferase, has been assessed in rats for hepatocellular injury, but it did not have any advantage over AST and ALT (Giffen et al., 2002).

There are few serum enzymes, originating from hepatocytes and biliary epithelial cells, that increase as a result of increased production secondary to intrahepatic or extrahepatic cholestasis and biliary proliferation. These include serum AP, γ-glutamyl transferase (GGT), leucine aminopeptidase (LAP), and 5′-nucleotidase (5'N). The most commonly used are AP and GGT. Both are bound to cytoplasmic and microsomal membranes. The mechanism for the cholestasis-induced production is uncertain, but bile acids are thought to stimulate enzyme synthesis.

Serum AP Activity

In dogs, serum AP activity is the most sensitive test for detecting cholestasis. It lacks specificity, however. Isoenzymes or isoforms of AP are produced by cells of the intestine, kidney cortex, liver, bone, placenta, and myeloid series. Normal serum AP activity in most adult animals is primarily the liver isoenzyme. Young, growing animals have an increased amount of the bone isoenzyme in serum secondary to increased osteoblast activity. In humans, but not dogs, placental isoenzyme is increased during pregnancy. The isoenzymes of liver and bone have half-lives of about 3 days in dogs, whereas those of intestine, kidney, and placenta are only 3–6 min. It is very unlikely that any of the latter three isoenzymes will produce serum elevations. Elevations in the bone isoenzyme rarely exceed three to five times the normal adult levels, even in young animals and animals with metabolic bone diseases. Corticosteroids and anticonvulsants can induce

liver isoenzyme production. Knowledge of the test material characteristics may help to identify probable drug-induced increases. In dogs, a unique steroid-induced isoenzyme can cause extremely high serum AP activity.

Because of cell swelling and pressure obstruction of small bile ductules, primary hepatocellular toxicities can cause intrahepatic cholestasis to elevate serum AP activity. Periportal lesions induce greater increases than do centrilobular lesions. Extrahepatic cholestasis, as might be seen with pancreatitis or biliary calculi, stimulates higher serum AP activity than intrahepatic cholestasis. Unfortunately, the degree of elevation is rarely sufficient to differentiate primary hepatocellular toxicity from primary biliary toxicity.

Serum GGT Activity

Serum GGT activity became popular because it purportedly had fewer interpretation problems than serum AP activity. Although its highest tissue concentrations are in the kidney and pancreas, serum elevations are reported to occur only with hepatobiliary lesions and certain drug therapies. Like AP, it can be induced by steroids and other drugs. Unlike AP, it is not affected by bone growth or disease. In most laboratory animal species, GGT appears somewhat less sensitive to hepatobiliary disease than AP, but it can be used for corroborative evidence. In rats, GGT is more useful than AP due to its intestinal isoenzyme in circulation that is influenced by food consumption and intestinal injury.

Serum LAP and 5′N activities have been investigated as alternatives to serum AP activity but have not found general acceptance. In some models of liver toxicity, 5′N appears to be more sensitive than AP.

The absence of change in serum hepatic enzyme activities does not signify an absence of hepatic toxicity. The liver can be severely dysfunctional and yet have little or no ongoing cellular degeneration. Elevations can be missed by poor timing of clinical pathology testing.

Bilirubin

Heme breakdown by cells of the mononuclear phagocyte system produces bilirubin. Hemoglobin from senescent erythrocytes provides about 85% of all bilirubin; hemoglobin from ineffective erythropoiesis provides another major source. Macrophage enzymes split hemoglobin into heme and globin, and heme is broken down into biliverdin and iron. Biliverdin reductase converts biliverdin to bilirubin, which is then released into circulation. At this point, the bilirubin is known as free, unconjugated, prehepatic, or indirect-reacting bilirubin. It is not water soluble and circulates bound to albumin. Hepatocytes remove unconjugated bilirubin from plasma and prepare it for removal from the body by a four-step process that includes uptake, conjugation, secretion, and excretion. Secretion of conjugated bilirubin across the canalicular membrane is the rate-limiting step in the process, and small amounts of conjugated or direct-reacting bilirubin escape into plasma, not bound to albumin. Conjugated bilirubin is freely filtered through the glomerulus. In most species, it is reabsorbed by the renal tubular epithelium, but in dogs, the renal threshold is low and traces of bilirubin are normal in concentrated urine.

The van den Bergh test differentiates unconjugated (or indirect) bilirubin from conjugated (or direct) bilirubin. The test is not very sensitive and should not be run unless the total bilirubin concentration is greater 2–3 mg/dL. It was designed to help distinguish prehepatic causes of hyperbilirubinemia from hepatic or posthepatic causes in clinical cases. In toxicology studies, however, the combination of clinical observations, other laboratory data (e.g., hematocrit and liver enzyme activities), and anatomic pathology findings are usually more than sufficient to determine the mechanism of hyperbilirubinemia. Laboratory determination of direct and indirect bilirubin is generally of little value.

Hyperbilirubinemia — Prehepatic or unconjugated hyperbilirubinemia is a very uncommon finding in toxicology studies. It occurs almost exclusively as a result of acute, moderate-to-severe hemolysis. If hepatocytes are unable to process the large amount of unconjugated bilirubin produced by the mononuclear phagocyte system during a hemolytic episode, there is an increase in total serum bilirubin concentration consisting primarily of the free form. It has been estimated that the healthy liver has the capacity to metabolize up to 30 times more free bilirubin than normal before hyperbilirubinemia results. A hemolytic episode sufficient to overload a normal liver and cause unconjugated hyperbilirubinemia will likely produce other evidence of hemolysis. Unconjugated hyperbilirubinemia may occur with less severe hemolysis in humans that have biochemical defect in the uptake and conjugation of free bilirubin. The Gunn rat is an animal model of defective conjugation.

Conjugated hyperbilirubinemia occurs as a result of impaired secretion of bilirubin, obstruction of bile outflow, or both. Because bilirubin secretion is the rate-limited step, any disease that damages the hepatocyte can potentially increase serum conjugated bilirubin concentration. Obstruction to bile flow may be either intrahepatic or extrahepatic origin. As with serum AP activity, periportal lesions induce greater hyperbilirubinemia than centrilobular lesions, and extrahepatic obstructions cause higher serum bilirubin concentration than do intrahepatic obstructions. In dogs, when hyperbilirubinemia is the result of a cholestatic process, serum AP activity is elevated.

Hyperbilirubinemia is most commonly due to increases in both conjugated and unconjugated bilirubins. For instance, severe hemolytic episode can cause secondary hepatocyte dysfunction due to hypoxia. The result is swollen hepatocytes that have impaired ability to secrete conjugated bilirubin. Alternately, biliary obstruction can cause secondary hepatocyte damage that may decrease the uptake and conjugation of free bilirubin.

Unlike hepatic enzyme activities, serum bilirubin concentration is a measure of liver function. In the absence of hemolysis, hyperbilirubinemia is an indication of liver dysfunction. In most examples of hepatotoxicity, however, there is normal total serum bilirubin because of the tremendous functional reserve of the liver. In dogs, where some conjugated bilirubin is excreted in urine, a 70% hepatectomy does not elevate serum bilirubin concentration.

It is interesting to note that human patients receiving anticonvulsant therapy have lower serum bilirubin levels than the population as a whole. Enzyme induction enhances the metabolism and excretion of bilirubin and can potentially mask an otherwise elevated bilirubin level.

Bile Acids

These are synthesized from cholesterol by hepatocytes, conjugated to an amino acid, secreted into the biliary system, and eventually excreted into the intestine. Bacteria transform some of the primary bile acids to secondary bile acids in the intestine. There is a very efficient enterohepatic circulation of bile acids, with the greatest part of the reabsorption occurring from the ileum. Portal blood returns bile acids to the liver for uptake, reconjugation, and resecretion. Any toxicity or drug that affects the liver can potentially alter one of the steps in the metabolism of bile acids and cause increased levels in serum. While not commonly used in toxicology studies, total serum bile acid concentration is sensitive and specific for hepatobiliary toxicity. By itself, however, total serum bile acid concentration does not provide enough information to discriminate between different types of hepatic lesions. Like serum bilirubin concentration, serum bile acids are a measure of a hepatic function.

Some additional markers of liver function include various proteins and substances synthesized by liver, such as glucose, cholesterol, urea nitrogen, and a variety of proteins, including coagulation factors. Severe hepatocellular dysfunction may cause decreased serum urea nitrogen concentration, hypoglycemia, hypocholesterolemia, hypoproteinemia (especially hypoalbuminemia), and prolonged coagulation times. On the other hand, liver disease may result in hypercholesterolemia and hyperglobulinemia. The pattern of changes elicited by liver toxicity is multifaceted.

Examination of the entire biochemical profile, along with hematologic and urinalysis findings, is necessary to properly evaluate liver toxicity.

Calcium and Inorganic Phosphorus

Calcium

Serum calcium concentration is affected by parathyroid hormone, calcitonin, and vitamin D and represents a balance between intestinal absorption, bone formation and reabsorption, and urinary excretion. Serum inorganic phosphorus concentration is affected by the same hormones but is more sensitive to dietary intake and urinary excretion. In order to interpret the changes in either of these parameters, it is helpful to know the results of the other.

Approximately 50% of serum calcium is ionized. As such, it is biologically active and participates in neuromuscular activity, bone formation, coagulation, and other biochemical mechanisms. Approximately 40% of serum calcium is bound to albumin in an inactive, unionized state. The remaining serum calcium is complexed to anions such as phosphate and citrate.

> *Hypercalcemia*. This is relatively uncommon in toxicology studies unless the test material, like some plant toxins, has properties of vitamin D. Miscellaneous causes of hypercalcemia include hypervitaminosis D, primary hyperparathyroidism, pseudohyperparathyroidism (i.e., secondary to some types of neoplasia), and occasionally renal disease.
>
> *Hypocalcemia*. Mildly decreased serum calcium concentration, secondary to hypoalbuminemia, is a frequent finding in toxicology studies. Signs of hypocalcemia do not occur because ionized calcium is relatively unaffected. Other miscellaneous and much less frequent causes of hypocalcemia include hypoparathyroidism, nutritional hyperparathyroidism, acute pancreatitis, puerperal tetany in the bitch, and renal disease.
>
> *Hyperphosphatemia and hypophosphatemia*. Increased serum inorganic phosphorus concentration is a normal finding in young animals. It may be as high or higher than serum calcium concentration. Serum inorganic phosphorus concentration is very sensitive to GFR and may be increased with prerenal, renal, or postrenal azotemia. Other less frequent causes of hyperphosphatemia include hypervitaminosis D, hypoparathyroidism, and nutritional hyperparathyroidism due to excess dietary phosphorus. Greatly decreased food consumption may cause hypophosphatemia, and this is sometimes observed in dietary toxicity studies if the animals refuse to eat.

Sodium, Potassium, and Chloride

Sodium is the major cation in serum and is the principle determinant of extracellular fluid volume (i.e., hydration status). Potassium is the major intracellular cation. Serum potassium concentration is maintained within narrow limits because of its critical role in neuromuscular and cardiac excitability. Chloride is the major anion in serum and serves to support fluid homeostasis and balance cation secretion.

While reference ranges for these parameters may appear fairly wide, the range of results in a well-controlled study is often quite narrow. Occasionally, very small statistically significant differences occur between control and treated groups in toxicology studies. Unfortunately, the mechanism for the difference is usually not apparent.

In most instances, serum sodium and chloride and concentrations tend to parallel each other. Hypernatremia is relatively rare, but hyperchloremia may sometimes occur secondary to secretory diarrhea with metabolic acidosis. In this condition, renal tubular reabsorption of chloride is increased because of decreased availability of bicarbonate. Decreased serum sodium and chloride concentrations can occur with gastrointestinal losses (e.g., vomiting or diarrhea), polyuric renal losses (e.g., chronic renal failure or diabetes mellitus), diuretics, and hypoadrenocorticism (a rare

finding in toxicology studies). Vomiting may cause hypochloremia and normonatremia because chloride, as hydrochloric acid, may be lost in excess of sodium.

Serum potassium concentration is a relatively poor indicator of total body potassium because of shifts between intracellular and extracellular compartments. Increased serum potassium concentration occurs with acidosis due to the exchange of extracellular hydrogen ions for intracellular potassium ions. Severe tissue necrosis and anuric or oliguric renal disease are infrequent causes of hyperkalemia. Decreased serum potassium concentration, like the changes in sodium and chloride, can be associated with gastrointestinal losses and polyuric renal losses. Potassium is also sensitive to decreased dietary intake and serum concentrations may be decreased along with inorganic phosphorus in animals that refuse to eat. Finally, an infrequent cause of hypokalemia is alkalosis.

Biomarkers and Nonroutine Assays

With the advancement of clinical pathology, biomarkers are getting increasingly popular in safety or toxicology studies. Biomarkers have been historically used for diagnostic purposes and toxicity assessment, such as ALT for liver injury, glucose monitoring for diabetes, and neutrophils for inflammatory conditions. However, recently, there has been tremendous effort on the development of new biomarkers for diagnosis of diseases as early as possible and to streamline and expedite drug development process.

There are numerous definitions of biomarkers but a more generic version is "characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic response(s) to a test article or therapeutic intervention." Biomarkers play an important role in drug development as they can assist in drug candidate selection, optimization, and confirmation of pharmacological response. Several new biomarkers are being investigated for detection of injury in various tissues such as heart, muscle, kidney, bone, pancreas, as well as some markers for process, e.g., inflammation.

Cardiac Biomarkers

Cardiac troponin, a biomarker of cardiac injury, is perhaps the most successful and ideal biomarker because it was developed first in human medicine and then utilized in animals and preclinical safety testing. Historically, LDH and creatine kinase isoenzyme (CK-MB) analyses were used in humans and animals to assess ischemia-induced cardiac injury. However, troponins (I and T), components of the contraction apparatus of striated muscle cells, are much more sensitive and specific markers for cardiac injury (degeneration/necrosis). Use of troponins has been recommended by the American College of Cardiology and the European Society of Cardiology for detection of acute myocardial infarction. Use of troponins has been expanding to assess cardiac injury associated with other disease processes as well, but it's not a direct marker of myocardial fibrosis or cardiomyocyte hypertrophy. There is considerable homology between human and nonhuman cardiac troponin isoforms, so many automated commercial human cardiac troponin immunoassays might work for nonhuman primates and other species (Reagan, 2010; Solter, 2007). However, species-specific assays will likely have better cross-reactivity and superior performance. The natriuretic peptides maintain cardiovascular homeostasis by increasing natriuresis, decreasing arterial blood pressure, and inhibiting the renin–angiotensin–aldosterone system. Atrial or A-type natriuretic peptide and brain or B-type natriuretic peptide have been utilized as markers for myocardial hypertrophy.

Skeletal Muscle Biomarkers

Conventional markers used for skeletal muscle injury, including AST, CK, and LDH, have very low sensitivity and specificity. In addition to these markers, aldolase has been used lately because

of its better performance, but more sensitive and reliable markers are still being explored. Utility of fast-twitch skeletal muscle troponin I (fsTnI) and urinary myoglobin (uMB) was evaluated as biomarkers of skeletal muscle injury in 8-week-old Sprague Dawley rats (Vassallo et al., 2009). Both of these markers had negligible/nondetectable concentrations in control animals and exhibited a dose-related increase in rats given various myotoxins. fsTnI was specific for muscle injury, but uMB was nonspecific and increased with skeletal muscle and cardiac toxicity. These biomarkers were concluded to have superior performance than traditional markers and recommended for use in conjunction with standard clinical assays for assessing drug-induced myopathy in rats.

Another panel of new biomarkers, comprised of skeletal muscle troponin (sTnI), fatty acid–binding protein 3 (FABP3), and myosin light chain 3 (MYL3), was compared in a comparative study against the traditional markers with various myotoxic and nonmyotoxic compounds (Tonomura et al., 2012). These new markers (sTnI, FABP3, and MYL3) appeared superior in the sense they had higher sensitivity and specificity and abundant distribution within the skeletal muscles and exhibited a positive correlation and higher diagnostic accuracy for detecting pathological alterations. However, they had several drawbacks that need to be considered including a rapid clearance, being affected by renal dysfunction, and different reactivity to various myotoxicants.

Renal Biomarkers

In 2008, seven urinary biomarkers, kidney injury molecule−1, clusterin, albumin, trefoil factor 3, total protein, cystatin C, and a2-microglobulin, were accepted by the Food and Drug Administration and European Medicines Agency for monitoring acute drug-induced renal injury in rat GLP studies. These biomarkers were to be utilized on a case-by-case basis under specific conditions when nephrotoxicity was expected and for monitoring renal safety in early clinical trials. Thus far, these assays are only available for rats and humans, and species-specific assay will be needed for other species because of issues with cross-reactivity (EMEA, 2009).

Bone Biomarkers

Serum and urinary assays of bone markers are noninvasive alternative to bone biopsy for assessing response to a disease or injury. The traditional imaging techniques, radiography and bone densitometry, only provide a snapshot of the bone mass at a given time but serum and urinary biomarkers quantify the formative and resorptive activities of bone in near real time. These assays provide a simple, sensitive, and accurate method of assessing disease progression or response to therapy. Markers of bone formation in serum include bone-specific AP, osteocalcin, and collagen, amino-terminal, and carboxy-terminal propeptides (PINP and PICP, respectively). Markers of bone resorption include fragments released from the amino- and carboxy-terminal regions of collagen type I termed as N-terminal telopeptide (NTX) and the C-terminal telopeptide (CTX) and their metabolic end products, free or peptide-bound deoxypyridinoline (Dpd) and pyridinoline. Of the two types, Dpd is considered more bone specific because it's not present in collagen type I from skin (Allen, 2003). NTX and CTX forms of collagen cross-links are found in serum and urine. Although NTX and CTX predominate, a third telopeptide, ICTP, which is a slight variant of CTX, is found in serum only. Because the bone formation markers (BALP, osteocalcin, and collagen propeptides) can only be measured in serum, serum would be the sample of choice for most clinical and preclinical studies. If urine samples also are available, they can be used to confirm any trends observed in the results of the serum assays for resorption.

Pancreatic Biomarkers

Insulin and glucagon, produced from alpha and beta cells of pancreatic islet cells, respectively, have been used as conventional markers for diabetes. Incretins are another class of hormones that

are being used for treatment as well as biomarkers for diabetes. Incretins are a group of gastrointestinal hormones that stimulate insulin release from islet cells in response to increased glucose. Since they only respond to increased glucose concentration, unintended consequence of hypoglycemia is not a concern with excessive dose or pharmacology. Incretins are rapidly degraded by enzyme dipeptidyl peptidase-4 (DPP-4). DPP-4 inhibitors are also used for treatment (Clause et al., 2007; Robertson, 2011).

Amylase and lipase have been used historically for detection of exocrine pancreatic injury, but they are poor indicators for early diagnosis of disease and prediction of disease severity. Interpretation of amylase and lipase is also complicated by their increase in nonspecific conditions. There has been renewed interest in markers of exocrine pancreatic injury due to incidence of pancreatitis in humans. The incidence of pancreatitis remains very low in preclinical safety studies making them poor predictors for pancreatitis in humans. Some new biomarkers are being explored for exocrine pancreatic injury (Walgren et al., 2007), but promising biomarkers have not been identified or adapted. In the mean time, a correlation of amylase and lipase with clinical signs remains the best option (Frank and Gottlieb, 1999).

Additional biomarker groups that are used depending on the known effects or mechanism of test material include thyroid hormones (thyroxine, triiodothyronine, and thyroid-stimulating hormone [TSH]), reproductive hormones, and inflammatory markers (acute-phase proteins, complement fractions, and cytokines). A few acute-phase proteins, such as C-reactive protein and fibrinogen, are being used more frequently because of the availability of assay and common occurrence of inflammatory responses in safety studies. However, discussion of these additional groups of biomarkers is more complex and will not be covered here.

Urinalysis

Urinalysis is part of the minimum laboratory database for clinically ill patients. It provides a specific evaluation of the urogenital tract as well as information concerning more generalized conditions. As a general observation, however, urinalysis is utilized improperly in many toxicology studies. For the most part, this is due to the technical difficulties associated with collecting a large number of urine specimens from small laboratory animals. The method by which urine is collected greatly influences the value and interpretation of the data obtained. If a toxin is known or suspected to affect the urinary system, measures can be taken to provide appropriate specimens for urinalysis (i.e., by catheterization, cystocentesis, or carefully collected voided samples). Usually, however, when a large number of animals are being tested with a test material of unknown toxic potential and regulatory guidelines require urinalysis, the most efficient method of urine collection (i.e., in a collection vessel at the bottom of a metabolic cage) produces a multitude of artifacts that diminish the value of the test results and may make interpretation impossible. Voided urine traverses the urethra, vagina or prepuce, and perineum or preputial hairs where it can acquire cells and bacteria. Added to the environmental contaminants in the bottom of a cage (e.g., cleaning chemicals, feces, bacteria, food, and hair) and given time (16–24 hours) to incubate, it is little wonder that urinalysis data from timed collections in a metabolic cage are less than desirable. Furthermore, it is nearly impossible to keep the collection vessel chilled during a prolonged collection, and preservatives such as toluene have disadvantages that preclude their use. The following discussion of the various parts of the urinalysis addresses the major issues of interpretation with respect to potential false-positive and false-negative results.

The urinalysis consists of two parts: physiochemical properties and sediment evaluation. The physiochemical properties include color, clarity, volume (for timed collections), specific gravity, and the reagent strip tests (pH, protein, glucose, ketones, bilirubin, urobilinogen, and occult blood). Some reagent strips have additional tests for nitrite (indicates presence of nitrite-producing bacteria) and leukocyte esterase, but these are not particularly valuable for animal specimens, especially

when the urine sediment is examined microscopically. Urinary enzyme activities, for the identification of renal tubule toxicity, are sometimes determined in investigational studies on test materials that are known or potential renal toxins.

Urinary sediment evaluation is a semiquantitative microscopic measure of the presence of cells, casts, bacteria, and crystals. In order to properly evaluate changes in the physiochemical properties of urine, it is important to know the urine sediment results. The reverse is also true.

Urine Volume and Specific Gravity

Timed urine volume and urine specific gravity are the only measure of renal function in the urinalysis. They demonstrate the urine-concentrating ability of the kidneys. The loss of urine-concentrating ability generally precedes development of azotemia as a consequence of primary renal disease. Urine specific gravity, as determined by refractometry, is an approximation of the solute concentration, but it is dependent on molecular size and weight as well as the total number of solute molecules. Urine specific gravity usually varies inversely with urine volume. Animals that have lost the ability to concentrate their urine have decreased urine specific gravity and increased urine volume. However, since urine specific gravity is a function of fluid intake, solute intake, glomerular filtration, renal tubular cell health, and other factors, the range of values that can be considered physiologically normal (e.g., 1.001–1.075) is quite large.

Hyposthenuria (i.e., urine specific gravity = 1.001–1.007) is sometimes observed in toxicology studies. Perhaps the most common cause is a leaky water bottle or automatic water system. More importantly, however, some nonhuman primates develop psychogenic polydipsia that results in excretion of very dilute urine. Because of high urine flow through the kidney, these animals may develop medullary washout and lose the ability to concentrate urine even if water is withheld. If water is unavailable, they can rapidly become dangerously dehydrated. For this and other reasons, it is probably inappropriate to withhold water from nonhuman primates. Unfortunately, some monkeys play with their water source habitually and make urinalysis results from cage-bottom specimens meaningless. There are many diseases that cause hyposthenuria, but these rarely occur in toxicology studies.

Isosthenuria (i.e., urine specific gravity = 1.008–1.012), also referred to as "fixed" specific gravity, occurs with advanced renal disease. Isosthenuria and hyposthenia are particularly meaningful when serum urea nitrogen concentration is elevated or the animal is dehydrated. If the urine sample is free of water contamination, this combination of findings indicates primary renal disease. Isosthenuria and hyposthenuria may also occur if the test material has diuretic activity.

When an animal is dehydrated and its kidneys are functioning properly, urine should be more concentrated than plasma. In toxicology studies, urine specific gravity is sometimes higher in treated groups than in control because the treated groups are not eating and drinking normally. Occasionally, test materials cause excessive gastrointestinal fluid losses that result in relative dehydration.

If water contamination of the urine sample is avoided, timed urine volume (e.g., 16 or 24 hours) and urine specific gravity are probably the most valuable urinalysis tests routinely performed. The other tests have too many complicating factors given the typical procedure for urine collection.

Reagent Strip Tests

Urine pH varies with diet; high-protein meat diets usually produce acid urine and cereal or vegetable diets usually produce alkaline urine. While test materials may alter urine pH, it is not a good indicator of acid–base balance. As urease-producing bacteria multiply in a standing urine sample, ammonia is produced and urine pH becomes more alkaline. Loss of carbon dioxide from specimens in open containers also causes urine pH to rise.

A small amount of urine protein, as measured with reagent strips, is a normal finding in most animals, especially if the urine is concentrated. A large number of proteins, especially in dilute urine, are abnormal. Increased protein excretion may be due to glomerular injury, defective tubular reabsorption, hemorrhage, inflammation, or the presence of proteinaceous material from the lower urogenital tract in voided specimens. The sediment findings may help to interpret the cause of proteinuria. The reagent strip reactions measure albumin better than globulin, so a false negative is possible in rare instances. The most common spurious findings are false positives owing to the effect of highly alkaline urine on the reagent strip reaction or contamination of the urine with quaternary ammonium compounds commonly used as disinfectants. Alternative methods of urine protein determination are sometimes used if these problems are suspected or if the urine protein result is expected to be an important piece of information.

Urine glucose is normally negative. Glucosuria is most commonly observed as a result of hyperglycemia (e.g., greater than 180 mg/dL in dogs) and the failure of the renal tubules to reabsorb the increased glucose load entering the glomerular filtrate. Diabetes mellitus is the most frequent disease associated with glucosuria. In toxicology studies, glucosuria is very rare, but it can occur with test materials that affect proximal tubular cells, decreasing their ability to reabsorb filtered glucose. False-negative findings for urine glucose can occur as a result of bacterial proliferation and consumption of glucose.

Ketonuria is occasionally observed in debilitated, anorectic animals and animals that have been fasted for a prolonged period of time. Ketonuria indicates that energy metabolism has shifted to incomplete oxidation of fatty acids. As might be expected, diabetic animals often have ketonuria. False-negative findings for urine ketones can occur as a result of bacterial degradation and the loss of volatile ketones from open containers.

Bilirubinuria, especially in concentrated urine, is a normal finding in dogs, but an abnormal finding in other species. Increased urine bilirubin occurs as a result of the same conditions that cause hyperbilirubinemia, but it often precedes the change in blood. False-negative findings for urine bilirubin can occur from prolonged exposure of the urine specimen to light that oxidizes the bilirubin to biliverdin.

Theoretically, urine urobilinogen tests the patency of the bile duct. Once conjugated bilirubin reaches the intestine, bacterial action converts some of it to urobilinogen, a portion of which is reabsorbed by the intestine. Most of the reabsorbed urobilinogen is removed by the liver, but a small amount is normally excreted in the urine. Therefore, a negative urine urobilinogen is supposed to indicate an obstructed bile duct. There are a multitude of problems with this test, and its value is questionable in any circumstance. It generally is determined simply because it exists on the same reagent strip as the other tests.

Positive findings for urine occult blood frequently occur in healthy animals. The origin of the blood is generally not known, but estrus in females is a common source. The reagent strips do not discriminate between erythrocytes, hemoglobin, and myoglobin. Results of this test must be correlated with the microscopic finding for proper interpretation. Hematuria occurs secondary to inflammation, trauma, or neoplasia of the urogenital tract and bleeding disorders.

Urine Sediment Evaluation

Small numbers of erythrocytes, leukocytes, and epithelial cells are normal findings in urine sediment. Large numbers, however, are abnormal. A variety of lesions can be responsible for hematuria and pyuria, at any level of the urogenital tract. Large epithelial cells (i.e., squamous and transitional cells) generally do not indicate serious abnormalities, but the presence of many small epithelial cells (i.e., renal tubular cells) is indicative of renal tubular lesions. Unfortunately, unless associated with granular or cellular casts, it is often difficult to distinguish renal tubular cells from other cells. Furthermore, prolonged exposure of all cells to the somewhat hostile environment of urine leads to

degenerative changes or cell lysis. This is one of the major problems with prolonged, timed urine collections. If sediment detail is important, other means of urine collection should be used.

Casts are cylindrical molds of protein and/or cells that form within the lumens of renal tubules. An occasional hyaline (i.e., proteinaceous) or granular cast can be a normal finding. An increased number of these casts or the presence of any cellular casts is an abnormal finding. Increased hyaline cast formation occurs when there is increased protein loss from the kidney. Cellular casts (erythrocyte, leukocyte, or epithelial) are rarely observed in animal urine but, if found, indicate a severe renal lesion. Cellular casts become granular casts as the cells within the cast degenerate. Granular casts, therefore, are also an indication of a renal lesion. Waxy casts are rarely seen. They represent the final stage of degeneration of the cellular cast and indicate prolonged, local or diffuse, intrarenal urine stasis. Broad casts are identified by their width and represent casts formed in collecting ducts or pathologically dilated portion of the nephron. As with waxy casts, broad casts indicate intrarenal urine stasis. While cells can originate all along the urogenital tract, cylindruria (increased number of casts) identified renal involvement. As with cells, casts degenerate with prolonged exposure to urine.

Because of the urine collection methods normally used, bacteria are a consistent finding in laboratory animal urine. In order for true bacteriuria to be confirmed, other collection methods must be used.

Crystals are also common findings in the urine of laboratory animals. The type of crystal is dependent on the urine pH. Triple phosphate, amorphous phosphate, calcium carbonate, and ammonium urate crystals are frequently seen in alkaline urine, and urate, oxalate, and hippurate crystals are associated with acid urine. Other types of crystals may be observed. A test material will occasionally form crystals in renal tubules or the development of calculi. Ammonium biurate crystals are associated with liver failure, and hippurate crystals are associated with ethylene glycol toxicity.

SPECIES DIFFERENCES

Species commonly used in safety studies are discussed first. General discussion from previous sections applies to all species. The following discussion focuses on distinct features/findings of each species.

Rats

Hematology

Rats (Table 12.1) have relatively small erythrocytes; MCV is generally between 50 and 60 femtoliters (fL) in adults. Central pallor is usually observed, and mild-to-moderate anisocytosis and polychromasia are normal findings that correspond to reticulocyte counts of 1%–3%. Howell–Jolly bodies and nucleated RBCs are occasionally observed. Crenation, fragmentation, and poikilocytosis of erythrocytes are commonly observed but are usually artifacts of slide preparation. Erythrocyte survival time is approximately 45–68 days. Hematocrits are generally between 40% and 50% and are slightly higher for males than for females.

WBC counts range from about 3,000 to 12,000/µL; counts for males tend to be slightly higher than for females. In young rats, lymphocytes comprise as many as 90% of the total cell count. With age, however, the neutrophil/lymphocyte ratio is closer to 40:60. Rat granulocytes sometimes have lobulated, ring-shaped nuclei. Immature granulocytes, such as band and metamyelocyte neutrophils, can appear as distinctive "doughnut" ring forms with a smooth nuclear membrane. Primary granules of the rat neutrophil are small and faint.

Inflammatory lesions in rats cause a neutrophilic leukocytosis, often accompanied by a lymphocytosis. Older rats with chronic lesions such as cage sores or tumors with ulcerated, infected

surfaces can have very high WBC counts (e.g., >50,000/μL) that must be distinguished from leukemia. A high percentage of Fischer-344 rats develop large granular lymphocyte leukemia after a year of age (Stromberg, 1985). In addition to high numbers of neoplastic cells in peripheral blood, the hematologic findings are also characterized by the development of an acute, immune-mediated hemolytic anemia exhibiting spherocytosis and reticulocytosis.

The rat spleen exhibits active hematopoiesis throughout life. In the bone marrow, the M/E ratio is usually between 1:1 and 1.5:1.0. Megakaryocytes are abundant, and lymphocytes are common, comprising up to 20% of the nucleated cell population. Mast cells are more prominent in rat bone marrow than in bone marrow from other laboratory animal species.

Platelet counts in rats are very high, averaging about 1,000,000/μL. Lower counts are frequently due to sampling-induced platelet aggregation, and platelet clumps are often observed on peripheral blood films. PT and partial thromboplastin time in rats are similar to other species.

Clinical Chemistry

In most respects, clinical chemistry findings and interpretations for the rat are not unusual. Because of the rat's prominence in biomedical research, however, there are numerous references in the literature covering a wide range of subjects relating to clinical chemistry. A high percentage of these report on the sources of variation such as the time of day when bled, method of handling, blood collection site, or anesthetic used. Several other references compare the merits of different tests for identifying lesions caused by various models of toxicity. For example, which of several liver enzymes is the best for identifying a specific model of liver toxicity? Rather than listing the details of these reports, some generalizations are made.

As with most species, serum glucose concentration is one of the most sensitive parameters to variations in handling and sample collection. Increased concentration due to experimental manipulations other than food consumption is often attributed to the effect of endogenous catecholamines and corticosteroids on glycogenolysis, gluconeogenesis, and insulin antagonism. While total serum cholesterol in rats is relatively resistant to atherogenic diets, a variety of disease conditions in older animals are associated with hypercholesterolemia. The reference range for cholesterol in aged rats is quite wide. In contrast to humans, HDL is the predominant lipoprotein in rats; the same is true of many other laboratory animal species. The γ-globulin fraction of serum protein determined by electrophoresis is remarkably low in the young adult rat, but as in most species, it increases with age of the animal and with exposure to antigenic stimulation.

In young adults, serum urea nitrogen and creatinine concentrations have narrow reference ranges. With age, however, the common occurrence of chronic progressive nephropathy, especially in males, affects the ranges.

Numerous liver enzymes have been studied and variously advocated for the study of hepatic toxicity in rats. In general, serum ALT, SDH, and GDH activities have been the best indicators of hepatocellular injury. GDH has been reported to be more effective biomarker of acute hepatocellular injury in rats than other liver enzymes (O'Brien et al., 2002). ALT has generally been adopted by most laboratories as the standard liver enzyme because it is easily available, less expensive than some nontraditional markers, and relatively easy to interpret. Induction of ALT occurs in rats with corticosteroid administration. However, the increase in serum ALT was much higher in proportion than the increases in liver ALT, suggesting that pharmacologically mediated induction of hepatic gluconeogenesis may have been a potential mechanism for increase in serum ALT activity (Ennulat et al, 2010a).

There has been considerable discussion concerning the relative merits of serum AP, GGT, and 5′N activities for the evaluation of hepatobiliary or cholestatic disease. It has been argued that serum AP activity is not specific or sensitive in rats and that changes in serum activity are due to changes in intestinal isoenzyme. In authors' experience, serum AP activity increases in association

with some hepatobiliary lesions and in the absence of visible histological gastrointestinal involvement. However, serum AP activity does not appear to be as sensitive to cholestasis in rats as it is in dogs. Furthermore, depending on the conditions of the experiment, serum AP activity has been shown to either increase or decrease secondary to food restriction (Oishi et al., 1979; Schwartz et al., 1973). In either case, the changes are small. Serum GGT activity is essentially nonexistent in normal rats, but a few studies have shown increased activity after treatment with hepatobiliary toxins (Ghys et al., 1975; Leonard et al., 1984). Serum 5′N has been used infrequently in rats but has been shown, in certain disease models, to parallel increases in serum AP and GGT activity (Kryszewski et al., 1973). In a retrospective study to assess diagnostic performance of liver enzymes in rats, diagnostic utility of AP and GGT was limited for biliary injury; however, ALP had modest diagnostic value for peroxisome proliferation. AT, AST, and total cholesterol had moderate diagnostic utility for phospholipidosis. None of the liver enzymes had diagnostic value for manifestations of liver hypertrophy, cytoplasmic rarefaction, inflammation, or lipidosis (Ennulat et al., 2010a).

Electrolyte findings are not notably different in rats. Like the erythrocytes of mice and humans, however, rat erythrocytes have high potassium concentration and hemolysis will yield spuriously high serum potassium values (Meeks, 1989).

The rat is commonly used as a test animal for organophosphate and carbamate products. Plasma cholinesterase activity in females is approximately three to four times higher than in males. While this difference is absent in very young animals, it becomes noticeable around 6–8 weeks of age. RBC cholinesterase activity is similar in males and females.

The primary adrenocorticosteroid in rats is corticosterone.

Urinalysis

Proteinuria is a common finding in rats, especially males, increasing with age and the development of chronic progressive nephropathy. Males begin to excrete low-molecular-weight sex-dependent proteins around 8 weeks of age (Alt et al., 1980). Larger proteins, including albumin, are excreted with the development of the nephropathy.

Mice

Hematology

Mice (Table 12.2) have the smallest erythrocytes of the common laboratory animal species; MCV is generally between 45 and 55 fL. Central pallor is usually observed, but because of the cell's small size, it is inconsistent. Moderate anisocytosis and polychromasia are normal findings and correspond to reticulocyte counts of 2%–5%. Mice have the highest reticulocyte counts among adult laboratory animals. Howell–Jolly bodies are a frequent normal finding, and nucleated RBCs are occasionally observed. Fragmentation and crenation of erythrocytes are commonly observed, but these are usually artifacts of slide preparation. Erythrocyte survival time is the shortest among common laboratory animals, approximately 40–50 days (Bannerman, 1983). Hematocrit is relatively high, especially if mice are fasted before sample collection. Although generally between 40% and 50%, hematocrits above 50% are frequently observed. Considering the small erythrocyte size, it is not surprising that mouse RBC counts are the highest encountered, ranging 8–11 million/mL.

Mice generally have WBC counts ranging from 2,000 to 10,000/μL; counts for males tend to be slightly higher than for females. Lymphocytes normally comprise about 70%–80% of the total cell count, but the percentage of neutrophils increases with age. Like the rat, mouse granulocytes often have lobulated, ring-shaped nuclei. When the ring is broken, the nuclei appear similar to those

of most mammals. As in rats, immature granulocytes appear as distinctive "doughnut" ring forms with a smooth nuclear membrane. In mice, neutrophil primary granules are small and very faint.

As with most species, inflammatory lesions in mice result in an increased absolute neutrophil count. Similar to the rat, however, this is often accompanied by an increase in absolute lymphocyte count. Older mice with chronic lesions may get remarkably high WBC counts, easily greater than 50,000/μL. Similar to rabbits, and in contrast to dogs, corticosteroids cause decreased absolute monocyte count in mice (Jain, 2000).

In addition to bone marrow, the mouse spleen exhibits active hematopoiesis throughout life. Erythropoiesis appears greater than granulopoiesis in the spleen, and the reverse is true in marrow where the average myeloid-to-erythroid ratio is usually around 1.5–1.0. Megakaryocytes are abundant in both locations. Lymphocytes are commonly found in bone marrow preparations from mice and may comprise up to 25% of the nucleated cell population.

Mice have the highest platelet counts encountered among laboratory animals. Counts normally range between 1 and 2 million/mL. Because sample collection is often problematic, it is not unusual to have lower counts due to platelet aggregation and to observe platelet clumps on peripheral blood films. PT and APTT in mice appear to be similar to other species, but these can vary because of several factors including methodology used, sample quality issues, and hydration status of animals.

Clinical Chemistry

The limited volume of available serum (or plasma) has a great impact on clinical chemistry procedures in mice. Even with the many advances in biomedical technology, it remains very difficult to complete a moderately full biochemical profile. It is nearly impossible if hematology tests are also desired. Evaporation during handling and testing can have a major effect or areas of concern. The number of tests measuring similar effects, such as urea nitrogen and creatinine for kidney dysfunction or multiple liver enzymes for hepatocellular injury, can be reduced to a single choice. Tests that exhibit large variation among individuals, such as electrolytes, may be of little value. If both hematology and a full biochemical profile are deemed necessary, then the number of animals tested should be increased so that individuals can be designated for one or the other test procedure. Pooling of samples for clinical chemistry analysis is not appropriate.

In general, clinical chemistry reference ranges for mice are quite wide. This is probably indicative of some of the difficulties associated with sample collection and analysis. Values for serum glucose and triglyceride concentrations are moderately higher than those normally observed with other species. Serum urea nitrogen values also tend to be higher, and concentrations as high as 40 mg/dL are common in normal mice.

Serum ALT and SDH activities are both useful for the detection of hepatocellular injury. Because serum AST and LDH activities are more easily influenced by muscle injury, such as occurs with handling or blood collection, these enzymes are less desirable if sample volume limitations are a concern. It is important to note, however, that serum ALT activity is also affected by handling. When compared with control mice that were either not handled or tail-handled 1 hour prior to blood collection, mice that were grasped by the body had serum ALT activity nearly four times greater (Swaim et al., 1985). An interesting enzyme activity phenomenon is associated with lactic dehydrogenase virus in mice. This viral infection induced decreased clearance serum enzymes resulting in elevations of LDH and AST activities, among others (Riley et al., 1978). In contrast to most laboratory animal species, salivary amylase is responsible for most of the serum amylase activity in mice and may increase with salivary gland injury.

The most notable electrolyte findings in mice are the reported reference ranges that are high and wide (e.g., 112–193 mEq/L for sodium; Everett and Harrison, 1983). It is likely that evaporation of the small samples has had some impact on the high end of these ranges. With respect to potassium,

mouse erythrocytes have a high intracellular concentration (similar to humans but in contrast to dogs), and hemolysis will cause spuriously high serum potassium concentration.

The primary adrenocorticosteroid in mice is corticosterone.

Dogs

Hematology

Dog (Table 12.3) erythrocytes are smaller than those of humans (MCV is generally between 60 and 75 fL), but the cells have the classic biconcave disc shape with an obvious area of central pallor. Because the normal reticulocyte count is less than 1%, anisocytosis and polychromasia are minimal. Howell–Jolly bodies and nucleated RBCs are rarely observed in normal adults. Sampling and slide preparation artifacts such as crenation, fragmentation, and poikilocytosis are less frequently observed in dogs than in smaller laboratory animals, especially rodents. Erythrocyte survival time is the longest of the common laboratory animals, approximately 100–120 days (Jain, 2000). Hematocrits are generally between 40% and 50% and are slightly higher for males than for females. Compared with most species, dog erythrocytes are relatively resistant to osmotic lysis as measured by osmotic fragility tests.

WBC counts range from about 5,000 to 15,000/μL. Laboratory-reared beagles tend to have lower and less variable counts than the general dog population. In contrast to the other species, neutrophils normally outnumber lymphocytes. The neutrophil/lymphocyte ratio is typically about 60:40–70:30. Dog neutrophils have segmented nuclei, and their primary granules are much fainter than those in human neutrophils.

Typical inflammatory lesions in dogs generally cause a neutrophilic leukocytosis. The presence of a left shift, as in all species, depends upon the severity of the lesion and the inciting agent. Lymphopenia, secondary to stress-induced endogenous corticosteroid, often occurs with serious inflammatory disease. In dogs, leukocyte counts greater than 50,000/μL occur with closed infections such as pyometra or prostatic abscess. Dogs typically respond to exogenous corticosteroid treatment with a mature neutrophilia, lymphopenia, eosinopenia, and monocytosis.

Hematopoiesis is confined to the bone marrow in normal dogs. Extramedullary hematopoiesis, usually in the spleen, may be observed in moderate-to-severe anemia with marked regeneration. The M/E ratio in the bone marrow usually ranges from 1:1 to 2:1. In contrast to rodents, lymphocytes generally comprise less than 5% of the nucleated cell population.

Platelet counts in dogs are generally in the range of 200,000–500,000/μL. Inherited factor VII deficiency, an autosomal recessive trait, occasionally affects beagle dogs from commercial breeding colonies. The disease is mild, causing no obvious bleeding problems with the exception of increased tendency to bruise. The dogs may also have increased susceptibility to systemic demodicosis. The disease is usually detected when PTs are measured in a group of dogs and one or more have consistently longer times. The difference is usually about 2–3 s.

Clinical Chemistry

The popularity of the dog as a pet is primarily responsible for the extensive database that has been accumulated concerning its clinical pathology findings in health and disease. The relative ease of sample collection eliminates many variables, such as collection site and anesthesia, that influence the interpretation of data from smaller animals. It is rather natural, therefore, to think of the dog as the "norm" and to compare other species with it. In this respect, clinical chemistry findings appear fairly straightforward. A few notable features of clinical chemistry in dogs will be discussed here.

When compared with the "street" population of dogs, laboratory-reared beagle dogs have very narrow ranges for several parameters, especially total protein, albumin, globulin, urea nitrogen,

creatinine, ALT, and AST. Carbohydrate metabolism in dogs is similar to that in humans, and glucose tolerance tests have been well documented (Kaneko, 2008). The range for total serum cholesterol concentration is higher and wider than for smaller laboratory species; values over 200 mg/dL are relatively common. Like many smaller laboratory species, values over 200 mg/dL are relatively common. Like many laboratory animals, but unlike humans, HDL is the most abundant lipoprotein.

Serum ALT activity is both sensitive and specific for hepatocellular injury, but as in other species, certain compounds may stimulate enzyme production and cause small increases in the absence of pathological change. Serum AP activity in dogs is very sensitive to intrahepatic or extrahepatic cholestasis but will increase with a variety of hepatic lesions. In addition, the dog has a unique steroid-induced AP isoenzyme that can be markedly elevated after administration of exogenous corticosteroids or secondary to hyperadrenocorticism. In these conditions, the hepatic isoenzyme activity will also increase. Serum GGT and 5′N activities parallel serum AP activity during cholestasis and are more specific for biliary diseases. The degree of change for GGT, however, is not as great as for AP. Decreasing activity of the osteoblastic isoenzyme of AP is responsible for the gradual lowering of serum AP activity with age. Serum GGT and 5′N activities are unaffected by changes in bone metabolism.

Total serum bilirubin concentration is slow to increase in cholestatic disease in dogs because the renal threshold for bilirubin is low. Bilirubinuria is a common finding, even for normal dogs.

Findings for electrolytes are similar to those in humans. However, because dog erythrocytes have a low intracellular potassium concentration, hemolysis does not cause spuriously high serum potassium values.

Thyroid and adrenocortical hormone tests are well documented (Kaneko, 2008). The size of the dog makes it a very suitable species for stimulation and suppression studies. Cortisol is the primary adrenocorticosteroid in dogs.

Urinalysis

As mentioned previously, bilirubinuria is a normal finding in dogs, especially in concentrated urine.

Nonhuman Primates

Hematology

Although the size of nonhuman primate (Table 12.4) erythrocytes is somewhat species dependent, they are generally smaller than those of humans. The MCV for cynomolgus monkeys varies according to their origin. Cynomolgus monkeys originating from China and Vietnam have larger erythrocytes (approximately 70–80 fL) than do those originating from Indonesia, Mauritius, or the Philippines (approximately 58–68 fL). The MCV for rhesus monkeys is similar to that for the cynomolgus monkeys from China and Vietnam.

Among laboratory animals, monkey erythrocytes tend to have the largest, most obvious area of central pallor. The cells often appear somewhat hypochromic, and MCHC is generally lower in monkeys than in other species. Values less 30% for MCHC are frequently observed. Because of these findings, there is a concern that low-grade iron deficiency may be a subclinical problem affecting laboratory primates. The normal reticulocyte count is less than 1%; anisocytosis and polychromasia are minimal. Howell–Jolly bodies and nucleated RBCs are rarely observed in normal adults. Erythrocyte survival time is approximately 85–100 days (Jain, 2000; Kreier, 1970).

Hematocrits are generally between 35% and 45% and tend to be higher for males than for females.

Subclinical malarial infection, recognized by the presence of intracellular *Plasmodium* trophozoites and schizonts, is relatively common in imported wild-caught or purpose-bred cynomolgus

and rhesus monkeys (Ameri, 2010; Donovan et al., 1983; Schofield et al., 1985; Stokes et al., 1983). Clinical disease is unusual without complicating factors such as disease-induced or drug-induced immunosuppression and splenectomy. Most frequently noted as an incidental finding on peripheral blood films of healthy animals, infections will occasionally cause an acute hemolytic anemia in animals secondary to experimental manipulations.

WBC counts range from about 5,000 to 20,000/μL. The extended reference range is mostly due to physiological leukocytosis observed in "untrained" animals bled without chemical restraint. The release of catecholamines may double the WBC count by mobilizing cells, both neutrophils and lymphocytes, from the marginal pool to the circulating pool. While this "alarm" or "fright" reaction can probably occur in any laboratory species, it appears to have the greatest effect on nonhuman primates. In nonhuman primates, lymphocytes are increased more than neutrophils during this reaction. The upper limit of reference ranges for anesthetized or trained animals is closer to 12,000/μL. The neutrophil/lymphocyte ratio is generally around 30:70 or 40:60. Neutrophil morphology is similar to that in humans; primary granules are prominent.

As in dogs, hematopoiesis is confined to the bone marrow. The M/E ratio is usually from 1:1 to 1.5:1. Lymphocytes generally comprise less than 5% of the nucleated cell population.

Platelet counts are generally in the range of 300,000–600,000/μL. Coagulation times, PT and APTT, are slightly longer than for dogs but similar to man.

Clinical Chemistry

As might be expected, clinical chemistry results for wild-caught monkeys are quite variable; the reference ranges for most parameters are broad. Although fasting serum glucose concentration is lower in monkeys than in other laboratory species, the "alarm" reaction can cause glucose values high enough to be confused with diabetes mellitus. High serum glucose concentrations (e.g., >150 mg/dL) should be checked with repeated serum glucose tests and urinalysis for glucose and ketones. Monkeys with diabetes mellitus frequently have high serum cholesterol and triglycerides concentrations. Atherogenic diets also cause increased serum cholesterol and triglyceride concentrations.

Wild-caught monkeys often have surprisingly high total serum protein and globulin concentrations; values over 9.0 and 5.0 g/dL, respectively, are not uncommon. The etiology is thought to be subclinical inflammatory or infectious disease with chronic antigenic stimulation causing a polyclonal gammopathy. Serum urea nitrogen and creatinine concentrations are higher and much more variable than in laboratory beagles.

Serum enzyme activities also tend to be highly variable. Increased serum activity of muscle enzymes (e.g., creatine kinase, AST, and LDH) may be observed secondary to iatrogenic muscle injury associated with handling or intramuscular injections of anesthetic. ALT, although present in muscle tissue in monkeys, is relatively specific and sensitive for hepatocellular injury. A fairly common cause of increased serum ALT activity in cynomolgus and rhesus monkeys is subclinical, enzootic hepatitis A infection (Slighter et al., 1988). Increased activity correlates with seroconversion to the virus and periportal inflammation. Because a percentage of animals entering a facility for use in toxicology studies are not already infected, exposure and infection may take place during the actual study period causing sporadic, high serum ALT activities in a few individuals. These results often confound the interpretation of the enzyme data.

Serum AP activity is much higher in cynomolgus monkeys than in any other laboratory animal species, and GGT may be of more value in the diagnosis of hepatobiliary disease in monkeys than in other species.

Values for serum electrolytes are extremely variable in unanesthetized monkeys. Serum sodium and chloride concentrations may range up to 170 and 125 mmol/L, respectively. The reason for these extremely high results is not known, but in the anesthetized animal, values over 155 and

115 mmL/L are unusual. In monkeys, serum potassium concentration may be spuriously elevated due to hemolysis since erythrocyte intracellular potassium concentration is high.

Cortisol is the primary adrenocorticosteroid in monkeys. Values in marmosets are higher than in macaques (Loeb and Quimby, 1999). The same is true of T_3 and T_4 (Kaack et al., 1979).

Rabbits

Hematology

Normal rabbit (Table 12.5) erythrocytes are slightly smaller than those of the dog; MCV is generally between 55 and 70 fL. The cells have central pallor and exhibit moderate anisocytosis and mild polychromasia that corresponds to a reticulocyte count of 1%–4%. Howell–Jolly bodies and nucleated RBCs are rarely observed on blood films from normal rabbits. The erythrocytes have a survival time of approximately 45–70 days. Hematocrit is generally in the range of 35%–45%.

Rabbit leukocytes have some unique features. The rabbit neutrophil, commonly referred to as a heterophil, is morphologically distinct from neutrophils of other mammals. Although the term *heterophil* has been used to describe the neutrophil of other laboratory animal species, the rabbit's heterophil is clearly unique. The cytoplasm contains many primary granules that are large, irregularly shaped, and darkly eosinophilic. Unless the cells are viewed in the same microscopic field, it may be difficult for the inexperienced observer to distinguish rabbit heterophils from eosinophils. Rabbit eosinophils have larger primary granules that are round and stain dull orange. The granules may partially obscure the eosinophil's bilobed or segmented nucleus. Rabbits are the only laboratory animal species to normally have circulating basophils counted during a standard differential WBC count. These cells have many dark purplish granules that obscure the nucleus. As many as 30% of the leukocytes in normal rabbits may be basophils.

The normal WBC count in rabbits, approximately 5,000–12,000/μL, is similar to the dog. The heterophil/lymphocyte ratio is approximately 1:2 in young adult animals and 1:1 in older animals. In response to acute inflammation, the rabbit's heterophil/lymphocyte ratio will increase, but in contrast to the dog, its WBC count may not (Toth and Krueger, 1989). Similar to most species, rabbits develop lymphopenia secondary to stress or exogenous corticosteroid administration. In addition, corticosteroids induce monocytopenia in rabbits.

A rare condition of rabbit leukocytes is Pelger–Huet anomaly. Observed in several species, including humans, this hereditary trait is characterized by failure of granulocyte nuclei to develop normal segmentation. In the heterozygous state, neutrophil morphology gives the appearance of a permanent left shift (i.e., the neutrophils always appear to be band forms), but the cells are not dysfunctional. Selective breeding in rabbits can produce a homozygous state that is invariably lethal.

Because normal serum calcium concentration is much higher in rabbits than other species, there is a potential for technical difficulties when performing coagulation tests. Calcium chelators such as citrate or oxalate are used for sample collection, and it is possible that overfilling collection tubes that have been prepared using the recommended anticoagulant to blood ratios may allow some fibrin formation to take place. If problems with tests such as PT, APTT, or thrombin time are encountered, consideration should be given to the sample collection method. For APTT evaluation, ellagic acid should be used as activator, and silica does not work in rabbits.

Clinical Chemistry

Fasting is recommended prior to blood collection for most species in order to standardize sampling conditions for the generation of, and comparison to, reference range values. For most experimental purposes, fasting rabbits for sample collection may be unnecessary because it appears to

have less effect on serum concentrations of parameters such as glucose and triglycerides than in other species. This may be due in part to the practice of coprophagy. Furthermore, the notable effect of fasting on rabbit body weight data may necessitate bleeding nonfasted animals in order to maintain proper conditions for other study objectives.

The most conspicuous biochemical differences between rabbits and other laboratory animals involve serum albumin and calcium concentrations. Albumin measurement by routine chemistry method (e.g., bromocresol green) using human or bovine standard works for most species including dog, rat, and mouse. However, the binding of bromocresol green to rabbit albumin is much greater and overestimates the concentration of albumin in rabbits by approximately 50% if rabbit albumin is not used as the standard or "calibrator" (Loeb, 2000). In fact, albumin concentration will often appear to exceed total serum protein concentration. Serum protein electrophoresis results indicate that albumin actually comprises about 60%–70% of serum protein. The cause of the spuriously high values determined with routine methods is not known. Results may be improved, however, by using a rabbit albumin standard for calibration.

Rabbits have higher total serum calcium than any other laboratory animals. While values as high as 16 mg/dL are not uncommon in healthy rabbits, they would clearly indicate severe hypercalcemia in other species. Conventional hypothesis was that high blood calcium in rabbits is due to excess dietary supplementation and, unlike other species, poorly regulated gastrointestinal absorption of calcium. However, these claims have not been supported by the recent research. Instead, it is speculated to be due to a "naturally high" set point for calcium-regulated parathyroid hormone (PTH) release (i.e., it takes higher concentration of blood calcium to turn off PTH production). Calcium excretion is also unique in rabbits where majority of calcium absorbed by the gastrointestinal tract is excreted via urinary tract. This differs from almost all other mammals where calcium is excreted, harmlessly, through fecal material (Rosenthal, 2004). Despite its relatively high serum calcium, mineralization of soft tissue lesions is not a prominent histological finding in rabbits. The VX_2 carcinoma of the rabbit is a model of tumor-associated hypercalcemia (Wolfe et al., 1978).

Serum cholesterol concentration varies more due to strain and gender than other parameters. As with many species, serum cholesterol concentration increases with age and is greater in females. Rabbits are more sensitive to dietary induction of hypercholesterolemia than most species and serve as a model to study atherosclerosis (Clarkson et al., 1974; Prasad, 2008). Cholesterol fractions in rabbits are normally comprised of approximately 45%–60% HDL, 30%–40% LDL, and 10%–15% VLDL. The majority of increase in cholesterol due to atherogenic diets is due to VLDL that can be 20–40-fold increased, compared with up to 4–5-fold increase in LDL (Campbell, 2004). Specific models for dyslipidemia (such as Watanabe heritable hyperlipidemic rabbits) can develop hyperlipidemia even when fed low-fat diet, free of cholesterol. Diet-induced hypercholesterolemia in rabbits is remarkable in that serum levels can reach as high as 3000 mg/dL. The serum in these animals is extremely lipemic, and this complicates serum biochemical analysis. Ultracentrifugation may be necessary to clear the serum samples.

ALT is not specific for liver injury in rabbits because of high ALT activity in muscles (Washington and Hoosier, 2012). However, ALT does not increase with restraint, and when treated with CCl_4, serum ALT activity is rapidly and markedly elevated.

The primary adrenocorticosteroid in rabbits is corticosterone. Rabbits are very sensitive to exogenous corticosteroids and develop typical signs even with topical or ocular administration and laboratory results for hyperadrenocorticism including hyperglycemia and increased serum aminotransferase activities. The latter is associated with diffuse vacuolization of hepatocytes.

Pancreas is the main source of amylase in rabbits with little contribution from salivary gland, intestines, or liver. Increased amylase in rabbits can occur due to pancreatitis, peritonitis, renal failure, or treatment with corticosteroids (Loeb, 2000; Washington and Hoosier, 2012).

Very low electrolytes can occur, usually in young rabbits, due to gastrointestinal loss. Varied symptoms can occur in enteritis including mucoid enteropathy. There are several predisposing

factors such as stress, high-carbohydrate diet or sudden dietary changes, or *E. coli* infection, and altered cecal flora is generally the underlying condition (Quinn, 2012).

Urinalysis

Because of its physical characteristics, rabbit urine is not routinely examined. The thick, turbid appearance is due to mucin and abundant calcium and triple phosphate crystals. The crystals may cause falsely elevated urine specific gravity if refractometric readings are made on samples that have not been centrifuged. Furthermore, the crystals make it difficult to visualize other formed elements in the urine sediment. In general, urinalysis is not recommended as part of the routine laboratory database in this species.

Hamster

Hematology

Many hematologic characteristics of hamsters (Table 12.6) are similar to other rodents, but there are a few differences. Their erythrocytes are morphologically similar to those of rats. The cells are approximately 50–60 fL in volume, and most exhibit some central pallor. Mild-to-moderate anisocytosis and polychromasia correspond to a reticulocyte count of about 1%–3% in the adult. Howell–Jolly bodies and nucleated RBCs are occasionally observed on blood films from normal adults. Erythrocyte survival time is approximately 60–70 days, but it increases greatly during hibernation and may reach as high as 160 days (Brock, 1960). Hematocrit is generally in the range of 35%–45%, and it also increases during hibernation. At the end of hibernation, reticulocyte production increases. Unlike mice and rats, the spleen does not contribute to normal erythropoiesis.

The normal WBC count is approximately 5,000–10,000/μL, and lymphocytes are the predominant nucleated cell, making up about 75% of the total. In contrast to RBC count, WBC count decreases during hibernation. Hamster neutrophils are similar to those of mice and rats and frequently appear in the peripheral blood as lobulated ring forms with fine, faintly eosinophilic primary granules. Band neutrophils and metamyelocytes have a distinctive ring or "doughnut" appearance with a smooth nuclear membrane. They are readily identified in bone marrow smear preparations. Although the normal myeloid-to-erythroid ratio has been reported as high as 10:1 in hamsters (Desai, 1968), it is probably closer to 2:1. Lymphocytes, a prominent finding in the bone marrow of other rodents and guinea pigs, do not represent more than a few percent of the marrow cell population.

While the clotting time for hamster whole blood is fairly rapid, PT and partial thromboplastin time are similar to those of most laboratory species (Desai, 1968; Dodds et al., 1977). Platelet counts in hamsters are not quite as high as in mice and rats, and the platelets are more difficult to see on peripheral blood films.

Clinical Chemistry

Serum glucose concentration in the hamster is similar to other species but is said to increase during hibernation (Lyman and Leduc, 1953) as do total serum protein and albumin concentration (South and Jeffay, 1958). Chinese hamster lines have been developed as models of insulin-dependent diabetes mellitus and as expected exhibit hyperglycemia, hypercholesterolemia, and hypoinsulinemia (Gerritsen, 1982). Of the small laboratory animals, hamsters appear to have the highest total serum cholesterol concentration (Carroll and Feldman, 1989). Amyloidosis is a common disease affecting older hamsters and similar to other species; hamsters with renal amyloidosis develop azotemia, hypoproteinemia, hypoalbuminemia, and hypercholesterolemia (Murphy et al., 1984).

Serum AP and ALT activities appear to be valuable as markers for liver injury, and serum LDH and creatine kinase activities increase in hereditary myopathy in Syrian hamsters (Homburger et al., 1996). Serum ALP activity is mostly comprised of bone isoenzyme in hamsters and only a small fraction (10%–15%) comes from intestines (Washington and Hoosier, 2012). The liver isoenzyme of ALP declines from weaning to undetectable levels in older hamsters.

The primary adrenocorticosteroid in hamsters is corticosterone, but cortisol is also present and will increase under the influence of ACTH (Albers et al., 1985; Ottenweller et al., 1985).

Guinea Pigs

Hematology

Guinea pig (Table 12.7) erythrocytes are the largest among common laboratory animals. Their MCV is generally between 75 and 90 fL, similar in size to human erythrocytes. The cells have central pallor and exhibit mild anisocytosis and polychromasia that corresponds to a reticulocyte count of 1%–2.5% Howell–Jolly bodies and nucleated RBCs are rarely observed. The erythrocytes have a survival time of approximately 80–90 days. Hematocrit is generally in the range of 40%–50%. An interesting phenomenon, unique to guinea pigs, is the development of an acute hemolytic anemia in response to high dietary cholesterol (Yamanaka et al., 1967). The belief is that alterations in membrane lipid content may render the erythrocytes more susceptible to lysis.

The most unique feature of guinea pig leukocytes is the Kurloff body, a cytoplasmic inclusion observed in a small number of mononuclear cells believed to be T lymphocytes because of their ability to form rosettes of rabbit erythrocytes (Jain, 2000). Kurloff bodies are a glycoprotein complex that generally appears as a single, large, round-to-oval, purplish red, homogenous inclusion. On occasion, a cell will have multiple smaller inclusions. Although the function of the Kurloff body is not known, they are observed more frequent in females than males, especially during the first 3 months of life, and appear to increase during pregnancy or with exogenous estrogen administration (Ledingham, 1940). In general, Kurloff bodies occur in less than 5% of circulating leukocytes.

Guinea pig neutrophils appear similar to those of humans in that the nuclei are distinctly segmented and the cytoplasm contains conspicuous reddish primary granules. The granules are not as large or prominent as those of the rabbit heterophil, but some authors refer to the guinea pig neutrophil as a heterophil. The cells are easily distinguished from eosinophils. In contrast to other species, guinea pig lymphocytes exhibit AP activity (Kaplow, 1969). This has significance because AP activity is sometimes used to cytochemically differentiate neutrophils that exhibit activity in most species, from monocytes and lymphocytes, which normally do not.

The normal WBC count in guinea pigs ranges from approximately 5,000 to 10,000/μL and will increase slightly with age. Regardless of age, the neutrophil/lymphocyte ratio remains approximately 1:2. Similar to mice and rats, as many as 25% of the cells in normal bone marrow of guinea pigs are lymphocytes. The typical myeloid/erythroid ratio is about 1.5:1.0.

With respect to coagulation, guinea pigs are unique among laboratory animals because of their relatively low concentration of the factor VII clotting protein. Consequently, one-stage PTs for the evaluation of the extrinsic clotting system are longer in guinea pigs, as long as 50–100 s in one study (Kaspareit et al., 1988). Automated coagulation analyzers that automatically stop if an assay has not begun to form fibrin within 50 s would be inappropriate for use on guinea pig samples. Thrombin time also appears to be relatively prolonged in guinea pigs. On the other hand, APTT is similar to other species.

Clinical Chemistry

There is not much information available concerning the clinical chemistry of guinea pigs. One of the most noteworthy items is that serum ALT activity is said to be insensitive and nonspecific as

a marker for hepatocellular injury. Part of this stems from the finding that the activity of ALT in other tissues such as muscle is as high as it is in the liver. In addition, liver ALT activity in guinea pigs is localized primarily within mitochondria and theoretically would be less likely than cytoplasmic enzyme to enter circulation secondary to hepatocellular damage. Regardless of these considerations, however, liver toxicity should remain the most likely interpretation for elevated serum ALT activity in guinea pigs. Normal serum ALT activity in guinea pigs is similar to that of other species.

Total serum cholesterol concentration will increase when guinea pigs are fed cholesterol-rich diets, but contrary to most species, the amount of HDL cholesterol also increases. Normally, guinea pigs have a smaller amount of HDL cholesterol that also increases. Normally, guinea pigs have a smaller amount of HDL cholesterol than other laboratory animals in humans. Their predominant lipoprotein is LDL.

The primary adrenocorticosteroid in guinea pigs is cortisol. While there is considerable homology among species with respect to the structure of insulin, guinea pig insulin is substantially different and is associated with differences in activity and receptor concentration (Zimmerman et al., 1974).

Ferret

Hematology

Compared with dogs, ferrets (Table 12.8) have relatively small erythrocytes; MCV is generally between 50 and 60 fL. Central pallor is usually observed. The cells exhibit only slight anisocytosis and polychromasia, even though reticulocyte counts have been reported as high as 12%–14% in normal ferrets (Thornton et al., 1979). Others report much lower reticulocyte counts, 0%–1%, which correlate better with the erythrocyte morphology (Sherrill and Gorham, 1985). Howell–Jolly bodies and nucleated RBCs are occasionally observed as might be expected since the spleen retains some hematopoietic capacity. Hematocrits tend to be slightly higher than those of the dog and generally range from 45% to 55%.

The WBC counts of the ferret range from about 5,000 to 15,000/μL. The neutrophil/lymphocyte ratio ranges from about 40:60 to 60:40. Leukocyte counts, morphology, and response to inflammation are similar to rats.

The most unusual feature of ferret hematology is estrus-induced bone marrow hypoplasia (Bernard et al., 1983; Kociba and Caputo, 1981; Sherrill and Gorham, 1985). Because the ferret is an induced ovulator, estrus may be prolonged if the female is not bred. Under the influence of endogenous estrogen, bone marrow hypoplasia develops in as many as 50% of the females with protracted estrus. The hematologic signs are characterized by an initial increase in platelet count and WBC count at the beginning of estrus followed later by pancytopenia (e.g., platelet count <50,000/μL, hematocrit <30%, WBC count <2,500/μL). The thrombocytopenia may be responsible for hemorrhaging that further lowers the hematocrit and leads to severe, fatal anemia. Systemic bacterial infections are sometimes associated with the leukopenia. Examination of bone marrow from severely affected animals reveals depletion of all hematopoietic cell precursors.

Clinical Chemistry

In general, clinical chemistry test results from normal ferrets more closely resemble those of the dog than any other common laboratory animal. Serum glucose, protein, and lipid concentrations are similar. For example, total serum cholesterol in the ferret and dog is relatively higher than that for most laboratory animals, but comparable to humans. Serum urea nitrogen and creatinine concentrations are similar to the dog. Serum ALT activity tends to be slightly higher than in dogs, but serum AP activity is somewhat lower. As with all other species, serum AP activity decreases with age. In contrast to mice, rats, hamsters, and monkeys, hemolysis does not spuriously increase serum

potassium concentration. Like the dog, it is thought that ferret erythrocytes have a low concentration of intracellular potassium (Lee et al., 1982).

Thyroid and adrenocortical hormone levels and their response to stimulation or suppression tests have been investigated (Garibaldi et al., 1988a,b; Heard et al., 1990). Resting values for serum thyroxine (T_4), triiodothyronine (T_3), and cortisol concentrations are similar to dogs. Stimulation with TSH causes increased T_4 but not T_3. Stimulation with ACTH, intravenously or intramuscularly, increases serum cortisol. Intravenous dexamethasone suppression (0.2 mg/animal) appears to decrease cortisol by greater than 50% at 3 and 5 hours postdose (Heard et al., 1990), whereas intramuscular dexamethasone (0.1 mg/kg) had little effect at 6 hours postdose (Garibaldi et al., 1988b). These findings seem to support the belief that the ferret is a relatively steroid-resistant species.

Urinalysis

Like the dog, bilirubinuria is a common finding. The threshold for bilirubin excretion is apparently lower in these species than in most others.

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Telemetry in Nonclinical Drug Safety Assessment Studies

R. Dustan Sarazan

CONTENTS

INTRODUCTION

Telemetry, as used in biology and medicine, can be defined as physiologic measurement that uses wireless data transmission from a subject to a recording apparatus. Its primary clinical and nonclinical application has been the cardiovascular system, although it is commonly used to monitor other organs systems as well. The use of telemetry for cardiovascular monitoring of unrestrained subjects had its origins in the early 1960s in San Diego, California (United States), through collaboration between Dean Franklin of the Scripps Clinic and Research Foundation (San Diego), Dr. Robert Van Citters of the University of Washington (Seattle) and Dr. Charles Schroeder of the San Diego Zoo veterinary hospital. *In vivo* telemetry technology has evolved dramatically in the intervening five decades since that experiment, due primarily to the development of modern miniaturized electronics, as well as biocompatible materials and advanced battery technology.

The majority of signals that are monitored *in vivo* by telemetry can be categorized as either a biopotential (electrical voltage) or a pressure. Telemetry devices differ in the number and combination of biopotential and pressure sensors they contain and in the anatomic locations where the sensors can be placed in the experimental subject. Additional signals commonly monitored by telemetry include temperature and position/motion. Blood flow and volume measurements can also be monitored by telemetry, but this is much less common.

Telemetry systems used in biomedical research can be either external or implantable:

External telemetry systems typically have a telemetry device that is carried in a garment pocket worn by the experimental subject with implanted or noninvasive sensors. A very familiar application in human medicine is an electrocardiogram (ECG) monitor worn by patients in a Cardiac Care Unit of a hospital. Patient ECGs are transmitted by telemetry to a monitoring station where the health of the patient can be monitored by medical staff. Similar devices have been developed specifically for nonclinical research applications such as drug safety assessment. These devices rely on digital wireless technology such as Bluetooth™ to transmit cardiovascular and/or respiratory parameters from experimental animals and have become widely accepted adjuncts to toxicology studies.

Implantable telemetry systems for nonclinical research have been in common usage since the mid-1980s. These devices rely heavily on modern technology such as efficient electronic circuits with low power requirements and self-contained batteries, similar to those used in modern hearing aids and implantable human pacemakers. Some implantable telemetry devices consume too much power for traditional batteries and use rechargeable batteries. Rechargeable implantable telemetry devices have not gained widespread acceptance in biomedical research to date.

HISTORY OF TELEMETRY IN NONCLINICAL DRUG SAFETY ASSESSMENT

Although implantable telemetry has been used in rodents by academic and drug discovery researchers since the 1980s, it was not widely adopted in drug safety assessment until the early 2000s. This adoption was largely driven by the public health issue of drug-induced electrocardiographic QT interval prolongation and subsequent fatal arrhythmias. The QT issue triggered the development of the *International Conference on Harmonization* (ICH) Safety Pharmacology guidelines, Topics S7A and S7B, finalized in 2001 and 2005, respectively. These documents required that blood pressure, heart rate, and the electrocardiogram be evaluated from nonrodent animal species prior to first human dosing of a new drug candidate. In addition, the S7A guideline specifically states that the electrocardiograms should be collected from conscious animals via telemetry.

The industry and regulatory response to the QT interval issue and the resultant ICH guidelines launched a new sub-discipline of nonclinical safety assessment known as Safety Pharmacology. In addition to standard repeat dose toxicology studies, separate acute nonrodent cardiovascular

telemetry studies then became a standard part of the nonclinical safety assessment program for new drug candidates. Studies with animal models chronically instrumented with telemetry largely replaced the terminal experiments in anesthetized dogs that had previously been used for drug discovery, efficacy, and safety testing.

Nonrodent telemetry studies proved to be powerful for the detection of electrocardiographic effects of drugs, primarily QT interval prolongation, and in combination with the development of the *in vitro* electrophysiologic hERG assay, effectively eliminated the risk of developing dangerous drugs like those that had previously caused patient deaths through this mechanism.

Although acute cardiovascular safety pharmacology telemetry studies using crossover experimental designs are very effective for small molecule drug candidates, they are not well suited for many large molecule drugs (biologics) such as monoclonal antibodies. These drugs are eliminated from the body very slowly and the typical crossover experimental designs used in safety pharmacology are not practical. Also, being foreign proteins, there is the risk of an immunological response to the molecule resulting in either the development of neutralizing antibodies that would interfere with subsequent exposures to the drug or an allergic response. In response, technology was developed to allow the collection of electrocardiograms from existing nonrodent repeat dose toxicology studies with external telemetry. Although toxicology study designs are inherently less sensitive than safety pharmacology study designs because they must use parallel instead of crossover protocols, the addition of external telemetry still provides the ability to detect large effects of biologics on the electrocardiogram. Subsequent hybrid telemetry systems, which combine external telemetry with extremely small implantable blood pressure sensors, have allowed the addition of accurate blood pressure assessment to toxicology studies as well.

PHYSIOLOGIC ENDPOINTS AVAILABLE BY TELEMETRY

Although the cardiovascular system is the most common application for telemetry in nonclinical safety assessment, the central nervous and respiratory systems can be evaluated by telemetry as well. These three organ systems are components of the "Core Battery" of organ systems that were deemed acutely critical for life in the ICH S7A Safety Pharmacology guideline and must therefore be studied *in vivo* prior to the first human exposure to an experimental drug candidate.

Cardiovascular System

The most common cardiovascular signals that are monitored in conscious experimental animals by telemetry are the electrocardiogram, systemic blood pressure, and cardiac left ventricular pressure. Additional cardiovascular signals that may be monitored by telemetry are pulmonary artery pressure and cardiac right ventricular pressure. A variety of derived parameters can be calculated from these signals by computerized data acquisition and analysis systems as shown in Table 13.1.

Respiratory System

Respiratory rate can be monitored by telemetry and it is typically derived from intrapleural pressure or diaphragmatic electromyogram signals but can also be extracted from an arterial pressure signal through digital signal processing in many animal species. However, a quantitative measurement of ventilation, such as tidal or minute volume (required by the ICH Topic S7A guideline), is considerably more difficult.

The only reliable method to measure tidal volume in unrestrained animals is through continuous measurement pulmonary air volume changes during the respiratory cycle. This can be achieved by monitoring the thoracic and abdominal circumference by elastic bands with variable inductance

Table 13.1 Parameters Derived from Cardiovascular Signals

Physiologic Signal	Derived Parameter
Electrocardiogram (ECG)	Heart rate
	PR interval
	QT interval
	QRS duration
	R-wave amplitude (uncommon for safety assessment studies)
	ST segment height (uncommon for safety assessment studies)
Arterial pressure	Systemic arterial pressure
	Pulmonary arterial pressure
	Pulse rate (may be different than heart rate)
	Systolic pressure
	Diastolic pressure
	Mean Arterial Pressure (MAP)
	Pulse pressure
Cardiac left ventricular pressure	Heart rate
	Systolic pressure
	Left ventricular end diastolic pressure (LVEDP)
	dP/dt_{max} and dP/dt_{min}[a]

[a] The peak positive and negative values of the first derivative of ventricular pressure (index of systolic and diastolic function, respectively).

using external jacketed telemetry or by implanted thoracic electrical impedance electrodes using implantable telemetry. The external bands are subject to some error due to positional changes of the experimental subject and variations in thoracic *vs.* abdominal respiration. Thoracic impedance technology measures changes in the relative quantity of air within the electrical field (lungs) and should, therefore, be more closely correlated with changes in lung volume during respiration, regardless of whether ventilation is produced by the diaphragm or the intercostal muscles (abdominal or thoracic breathing, respectively).

Central Nervous System

The most common application of telemetry to the nervous system in nonclinical drug safety assessment is the electroencephalogram, or EEG. These biopotential signals are typically measured through electrodes inserted in the cranium over the surface of the brain. Multiple pairs of electrodes may be placed in various configurations to evaluate brain wave activity in different regions of the brain. A pair of electromyogram (EMG) electrodes may be used simultaneously with EEG electrodes to eliminate noise due to biopotentials created by skeletal muscle contractions in the head and neck.

EEGs from experimental animals are useful for detecting premonitory signs of seizure activity in drug safety assessment as well as for sleep research in drug discovery and efficacy pharmacology studies.

Other Applications

In addition to the cardiovascular and central nervous systems, telemetry is used less frequently to collect biopotential signals from skeletal muscle (EMG) and nerves (eg: sympathetic nerve activity, or SNA). Specific measurement of ocular muscle biopotentials is called an electrooculogram (EOG). EOGs are particularly useful for detecting and quantifying rapid eye movement (REM) during sleep.

Newer applications of telemetry that are under development or investigation at the time of this writing include blood glucose, neurotransmitters in the brain (glutamate, acetylcholine, etc), and blood pH, oxygen and CO_2. Glucose, for example uses enzymatic/electrochemical sensors incorporating glucose oxidase enzyme. This enzyme reacts with glucose to produce hydrogen peroxide, which creates an electrical current on a platinum wire substrate. This current is directly proportional to the amount of glucose present at the sensor and can be measured with a simple electronic circuit.

The technical requirements to interface many of these various chemical sensors with a telemetry device are not overly challenging. The greatest challenge to chronic measurement is the stability of the enzymes in the sensor and the biologic interaction with the surround tissue or blood (biofouling). Many available enzymes are not inherently stable *in vivo*, although various methods to improve the enzyme stability and biocompatibility of sensors are under development.

CHARACTERISTICS OF IMPLANTABLE TELEMETRY SYSTEMS

Nonclinical telemetry systems used in small and large laboratory animals vary in several respects. A general understanding of these differences is required for a knowledgeable evaluation and selection process before implementing telemetry in a laboratory. Most options have tradeoffs that should be understood.

Battery Technology

The options for battery technology include single use batteries that are contained within the telemetry implant, a battery module that can be disconnected from the telemetry implant and replaced through a minor surgical procedure, and rechargeable batteries that can be inductively recharged by maintaining the animal and a radiating energy source in close proximity.

By far the most common approach in small animal implantable telemetry is single use batteries that are contained inside the telemetry implant. This is also the approach used by the leading manufacturers of large animal telemetry devices. By using low power electronics and turning the device off when data are not actually being collected, these devices can often last longer than the expected lifespan of the experimental animal. Newer digital products have the ability to monitor and calculate remaining battery life so an investigator knows how much data transmission time remains for a device before assigning an animal to a study. This ensures that the animal will produce data until the study is complete. In addition, some digital products automatically enter a low power standby mode or even turn themselves off when they detect that they are not in communication with a data acquisition system. This is useful since the most common cause of premature loss of a battery, and therefore a telemetry implant and its host animal, is being accidentally left on by laboratory personnel when not needed. In cases where animals must be used for very long periods of time (e.g. 12 months or more), including the collection of large amounts of continuous data, a separate removable battery module is an option. This approach is commonly used in rodent CNS research where EEGs must be collected for 12 months or longer.

The third option of using a rechargeable battery has had some success, although limited to small animals only. An advantage is that more power can be consumed, which can allow for high power consumption applications such as increased transmission range. Another proposed advantage is that the devices can be explanted and presumably reused indefinitely. Whereas this can be done occasionally, in practice it is very difficult to dissect implanted devices out of an animal without damaging the sensors. If damaged, the entire device is typically discarded and replaced with a new unit. Other disadvantages of the rechargeable technology are that the amount of continuous data that can be collected between recharges is often limited due to high power consumption and the short time interval between charges. Also, inductive recharging by nature generates heat, which

is difficult to control in a freely moving animal if recharging is necessary during data collection. This would be especially problematic if temperature was being collected from the device during the recharging process.

Considerations for Product Reuse

Implantable telemetry devices used in short term studies, typically in rodents, are often carefully explanted, cleaned, tested, disinfected and reimplanted into another animal for reuse. This can be done repeatedly until the end of the functional lifespan of the particular device. They must then be discarded or returned to the manufacturer and replaced with new ones. This functional lifespan varies depending on (1) its ability to maintain consistent calibrations and drift over time *in vivo* (2) the ability of the device to prevent moisture ingress from the *in vivo* environment, and (3) the combined circuitry and battery technology design (battery life).

Between implant reuse it is important to check the accuracy of the calibrations on the bench. Pressure sensors drift over time, moisture ingress can cause circuits to fail and battery life should be checked and recorded prior to reimplantation into a new animal. To help researchers reduce ongoing study costs, some manufacturers offer an exchange program or recalibration service for implants. The exchange or recalibration cost can be dramatically less than the cost of a new device. Although purchase arrangements vary according to a given manufacturer's business model, the devices are typically purchased at the full price initially (often part of a system startup capital expenditure) and the much lower price of the exchanged units is considered an ongoing study expense.

There are some reasons why returning devices to the manufacturer may be impractical or even unsafe to personnel who handle them at the laboratory site and at the manufacturer. An example of the latter may be high biocontainment safety level research (BSL-4) with deadly pathogens or chemicals such as those that are studied in biological or chemical defense research.

Transmission Range and Tissue Penetration

The transmission range of implanted telemetry devices depends on several factors including transmitter power consumption, amount of tissue between the implant and the external environment, the size of the antenna, and the radio frequency (RF) used for transmission.

As with commercial radio stations, the amount of power used to drive the antenna directly affects the transmission distance that can be covered by the signal. However, with implantable telemetry, high power consumption comes at the cost of shortened battery life.

Signal attenuation from penetration through animal tissue also affects the distance that a signal can be transmitted between an animal and a receiver or transceiver. The size of an experimental subject and the location of the implant within the body are obvious factors that affect the amount tissue that must be penetrated and therefore the signal loss between the implant's antenna and the outside environment. The radio frequency (RF) used by the system is also very important. Lower radio frequencies (<1 MHz) tend to penetrate animal tissue with relatively low signal loss. Conversely, higher radio frequencies (>1 GHz) tend to be attenuated very easily by animal tissue.

Development of a successful telemetry system requires optimization of transmitter power, radio frequency selection, and antenna design. In addition, depending on the size of the experimental animal and the transmission distance required for the laboratory housing conditions, the location of the device within the animal should be considered.

In the case of rodent telemetry, the cage can be placed immediately on top of the antenna and the rodents always reside in the bottom of their cage, making the transmission distance quite small. In this application a long transmission range would be considered deleterious since cages would have to be widely spaced or shielded to prevent interference from the adjacent animal's transmitter. Thus, the dramatic success of research using implantable telemetry in rodents during the past two

decades was achieved by the selection of a relatively low radio frequency (455 KHz) and low transmission power settings, offering very long implanted battery life.

The same technology that is used in rodents has also been successfully used for research in larger animals, such as dogs and nonhuman primates for many years by placing one or two receivers inside each animal's cage. However, recently developed animal husbandry requirements require larger cages with multiple animals together in social housing arrangements, making the original technology less appropriate for large animal telemetry studies. Fortunately, the evolution of digital electronics technology and the use of higher radio frequencies (approximately 900 MHz) allow for longer transmission ranges while still limiting tissue attenuation and also providing the ability to monitor multiple animals simultaneously without interference or crosstalk.

A very important, but often overlooked, consideration in the selection of telemetry equipment is to confirm that the system operates in a radio frequency band that is approved for use by the government in the country where the system resides. In the United States this is regulated by the *Federal Communications Commission* (FCC). Given that these systems are typically used inside a vivarium space and the transmitted signal may not extend beyond the building, some have ignored this consideration in the past. However, in a highly regulated environment like Good Laboratory Practice (GLP) compliant nonclinical safety assessment, violation of government regulations can have consequences for the laboratory. Also, the radio frequency spectra inside vivaria are becoming much more crowded with the introduction of other wireless technologies such as cellular, Wi-Fi, Bluetooth, and Zigbee. By ensuring that a telemetry system operates within an approved radio frequency band, interference between these wireless technologies can be reduced or eliminated.

Single vs. Group (Social) Housing

Conscious animal research has evolved from the study of animals in individual cages to combining two or more animals in the same cage. This is a more natural environment for most experimental animals and has been demonstrated to lower animal stress, as indirectly measured by blood pressure and heart rate. In addition to improving the quality of the science, this is a desired improvement in animal welfare practices and is, in fact, becoming a regulatory requirement in many regions of the world.

With these changes in animal husbandry practices, telemetry technology has evolved as well. Telemetry platforms come with single or multiple frequency communication protocols. In the latter case, systems can monitor more than one animal at a time. For most researchers, it is only necessary to collect data from one of the animals in the cage at a time, but in situations where behavior may impact physiology or in studies looking at the transfer of disease from one animal to the next, multiple frequency telemetry units may be necessary.

Rodent Housing

Telemetry studies in small animals, such as rats, mice and guinea pigs, have evolved over the years to be more commonly conducted with pair housed animals whenever possible. The original implantable rodent telemetry technology that was developed in the 1980s operates on a single radio frequency with a low power transmitter. When used with pair housed rodents these studies required staggering of data collection such that only one animal's transmitter is turned on at a time.

This arrangement is not ideal for the efficient conduct of studies and telemetry technology is evolving to meet these new husbandry requirements. The combination of multiple frequency devices, scheduled sampling protocols and the addition of digital two-way communication ensures that multiple signals can be simultaneously collected from the same cage. The possibility of signal interference (cross talk) between the animals' devices is eliminated by absolute animal and signal identification by the data acquisition system. Newer digital technologies have become critical for

ensuring data integrity whether a study is conducted as a GLP safety study or in a basic research setting where results may be published in the peer reviewed literature or used to support intellectual property claims in a subsequent patent filing.

Large Animal Housing

The functional effects of new drug candidates on the cardiovascular system in conscious large animals began to be extensively studied by the pharmaceutical industry in the early 1990s, primarily by using implanted devices with external wires and tethers. Although external telemetry had existed since the 1960s, there were no commercially viable implantable solutions until well into the 1990s. Implantable telemetry in large animals experienced rapid and widespread adoption by pharmaceutical companies and their *Contract Research Organization* (CRO) partners as a response to the public health concern over drugs that prolong the electrocardiographic QT interval and the subsequent global adoption of the ICH S7A&B regulatory guidelines between 2001 and 2005.

Large animal telemetry studies were initially performed with animals separately housed in individual cages. This was a carryover from the tether and swivel methodology and due to limitations of the telemetry systems and the nature of the studies themselves. Animals were kept separate for dosing purposes to ensure that each animal received only its own test article and none could be consumed by a cage mate following regurgitation or from feces. In fact, animal welfare guidelines at the time allowed a specific exception from social housing requirements during data collection by telemetry.

More recently, expectations for social housing of large animals, especially nonhuman primates depicted in Figure 13.1, began to increase even for telemetry studies. This was driven primarily by the development of European animal husbandry and welfare guidelines in the 2000s. The Europeans were the leaders in the development of social housing for large animals, including those in telemetry studies, while very little activity occurred in the United States where there were no similar animal welfare regulatory requirements. However, in recent years, large multinational pharmaceutical companies and the CROs who conduct studies for them began to expect European style husbandry practices to be implemented in the United States as well, in spite of a lack of regulatory requirements by the *United States Animal Welfare Act* and therefore a lack of enforcement by the *United States Department of Agriculture* (USDA).

In response, contemporary large animal implantable telemetry systems are capable of collecting cardiovascular signals from a number of experimental animals that are group housed in the same room or even the same cage. Some are fully digital systems, comparable to modern cell phone technology, which provide absolute animal identification, auto-calibration, auto configuration of software systems and real time energy management and battery life monitoring. These digital systems operate at radio frequencies that provide an optimal compromise of tissue penetration, transmission range, battery life and are within the legal radio frequency spectrum for the host country.

Sensor Technology

Although all of the preceding characteristics of a telemetry system are important, none of them matter if the sensor that is capturing the physiologic signal is of insufficient quality. In computer science this is referred to as "Garbage In, Garbage Out." Faithfully reproducing an inaccurately measured or poor-quality signal with sophisticated electronic technology is of little value to scientists, drug companies, or regulatory agencies.

As was stated in the section "Introduction," the four sensor types that are commonly used in telemetry devices used for nonclinical research are: pressure, biopotential, temperature, and motor activity. Each of these sensors will be discussed individually below.
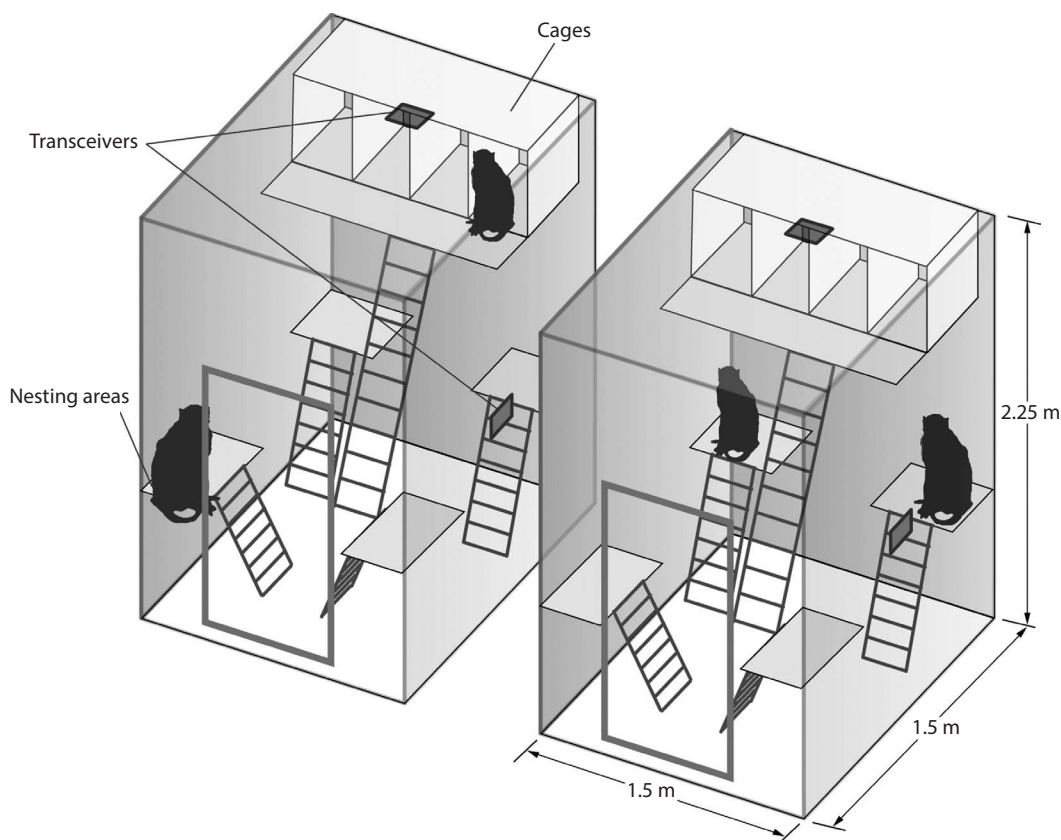
Figure 13.1 Group housing of large animals: Advances in telemetry technology allow multiple animals to be housed in a single unit. Transceivers are distributed throughout the caging environment and by using unique radio frequencies that are paired with "smart" digital technology, animals can be continuously monitored without the risk of data corruption or loss due to "cross-talk."

Pressure

Pressure sensor technology used in most physiology research today operates on the principle of the strain gauge and the Wheatstone bridge electrical circuit (see Figure 13.2). Although very early devices used a variable inductance method, these were replaced by the strain gauge systems in the 1950s and 1960s. The strain gauge was invented in 1876, was refined into a fluid pressure sensor by Louis Statham in 1943 and was first used to measure blood pressure at the Mayo Clinic in 1945. Although the materials used to manufacture pressure sensors are much improved, and have been dramatically miniaturized, the principle of operation of the devices used in modern implantable telemetry systems is virtually unchanged from the original one from decades ago.

The sensor is simply a thin diaphragm that is exposed to the pressure of interest on one side and is either vented to the atmosphere or a sealed reference chamber on the other side. As the pressure of interest increases (blood pressure, for example), this places stress on the diaphragm and it is deflected away (strain) from the increased pressure. The magnitude of the deflection is determined by the amount of pressure differential across the diaphragm and the stiffness (or compliance) of the diaphragm. The strain of the diaphragm is converted to an electrical potential proportional to the pressure by four small transducers attached to the diaphragm. They each increase or decrease their resistance proportional to the strain. A fixed input voltage is provided to the strain gauge and the
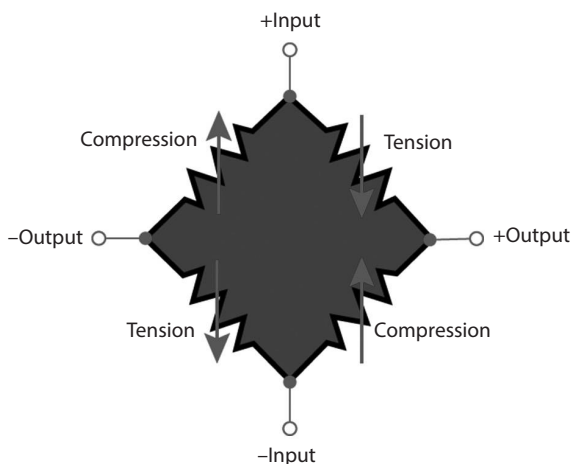
Figure 13.2 Wheatstone bridge: The bridge is connected to a thin membrane that flexes with changes in pressure. Changes in resistance occur when the resistors are under compression or tension. Composed of four resistors, the bridge is capable of detecting changes in compression and stretch that can be used to represent changes in pressure.

changes in resistance in the four small transducers result in an output voltage that is proportional to the diaphragm strain and therefore the stress, or pressure.

Although the principle behind the strain gauge pressure transducer is simple and has been unchanged for several decades, several advancements have been made to improve stability with respect to temperature and time (drift), as well as miniaturization to allow implantation inside smaller and smaller animals, such as mice.

In vivo pressure measurements were almost exclusively performed by scientists with the Statham (later Gould) strain gauge pressure transducer in the 1950s and 1960s by attaching a fluid-filled plastic catheter to a dome on one side of the strain gauge diaphragm while the opposite end of the catheter was inserted into the blood vessel, heart chamber or other structure, of interest. The device, pictured in Figure 13.3, was easily calibrated before each use with an external mercury manometer such as was used with human cuff blood pressure measuring devices (sphygmomanometry). This allowed pressure measurements with the conversion of the millivolt outputs of the Wheatstone bridge circuit into physiological units (mmHg).

This old apparatus was the mainstay of nonclinical as well as clinical research for several years and is responsible for vast improvements in our understanding of the cardiovascular system by our scientist predecessors. However, the method had significant limitations. First, the subject was required to remain motionless since the transducer was outside the body and connected by the catheter. This required physical or chemical restraint in nonclinical studies. Second, the pressure measured depended on the relative height of the external transducer compared to the site of interest for measuring pressure, which was the other end of the catheter. This hydrostatic pressure (or head pressure) effect was such that raising the transducer with respect to the subject would lower the indicated pressure, or lowering it would increase it, even though there was no real change in the actual pressure being measured. The inverse was true for movements of the experimental subject with respect to the fixed pressure transducer; if the subject moved up, the indicated pressure artificially increased, etc. Third, depending on the length, diameter, and compliance of the catheter, a delay between the occurrence of a pressure event and its detection at the external transducer could occur. This is referred to as "phase shift." Fourth, and most important, the bandwidth (frequency response) of the pressure measuring system was limited. This was also dependent on the length, diameter, and compliance of the catheter.

Figure 13.3 Statham pressure transducer: The pictured transducer has a thin metal membrane that is connected to a Wheatstone bridge. The plastic dome covers the membrane and allows connection of a fluid-filled cannula and a flush port to remove all bubbles from the system and keep the cannula clear of blood clots.

The first practical implantable pressure transducer for physiology research was designed by Robert Van Citters and Dean Franklin in 1965 and was used in a famous telemetry study of wild baboons and giraffes in Africa the same year. The wires from this prototype transducer exited the animal's skin after surgical implantation and were connected to the supporting electronics worn externally by the animal. With this device, the metal diaphragm with the four strain gauge transducers attached inside, was inserted into the aorta or cardiac left ventricle of the animal, thus entirely eliminating the issues with the long fluid-filled catheters used with the external Statham devices. The most notable problem with these devices was that they tended to drift over time and had to periodically be recalibrated. This was often done with a simultaneous external pressure sensor, usually a Statham device with a co-located fluid-filled catheter while the animal was briefly restrained.

Subsequently, a similar but smaller version of the device was developed where half of the bridge transducer was miniaturized to fit in the end of a catheter that could be inserted into a vessel percutaneously. These half-bridge catheter-tipped transducers were developed for acute, short-term use in humans, were calibrated prior to insertion each time, and were not optimized for nonclinical researchers or for chronic use at the time.

The first pressure sensing system developed explicitly for totally implantable telemetry was invented by two engineers, Brian Brockway and Perry Mills, in St. Paul Minnesota (United States) in 1987. It was developed for academic researchers desiring to measure arterial blood pressure in unrestrained, untethered, conscious rats. This device contained a micromachined silicon bridge transducer that was located inside the sealed body of the telemetry device, where it was protected along with the battery and the electronics. The pressure transducer was coupled to the intravascular site of interest by a short catheter that was filled with a proprietary incompressible fluid. The length, diameter, and compliance of the catheter were optimized to provide a signal bandwidth adequate to faithfully measure pulsatile pressure in the rat aorta with minimal phase shift. By using a larger (full-bridge) strain gauge transducer that is protected inside the housing of the transmitter, exceptional stability with respect to long-term drift was achieved (<2 mmHg/month). This same system, pictured in Figure 13.4, has been in widespread use in rodent telemetry since 1988 and was
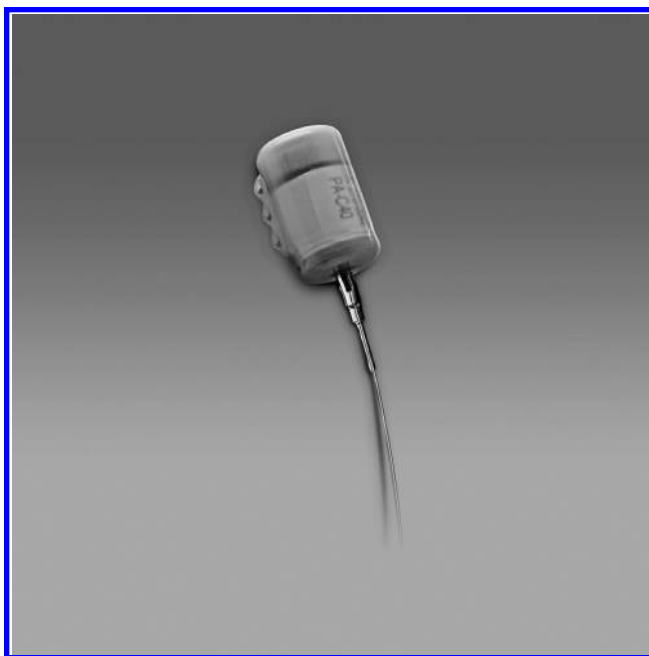
Figure 13.4 Rodent pressure transmitter: The device originally developed in 1988 is still used today for measuring a single pressure, temperature and activity.

subsequently adapted for use in large animals as well. Through improvements in sensor technology and improved compliance characteristics of the pressure transmission catheters, these devices have now been shown to have optimal bandwidth characteristics to accurately measure arterial blood pressure as well as cardiac left ventricular pressure (LVP) in rats, dogs, minipigs, and nonhuman primates, including the index of ventricular contractility, dP/dt_{max}. This is achieved for long durations in an animal with minimal drift and no need to recalibrate the devices *in vivo.*

Although the most common use of pressure sensors in nonclinical telemetry studies is in the cardiovascular system, pressure in other portions of the body are also commonly measured. Examples include the urinary bladder, ventricles of the brain, the eyes, tumors, and the reproductive organs.

The most commonly discussed, and misunderstood, characteristic of an *in vivo* pressure sensor is its bandwidth (often referred to as frequency response). A comprehensive discussion of this subject is provided in the section "Suggested Readings" and is beyond the scope of this chapter. Suffice it to say the high end of the bandwidth (frequency response or low pass filter cutoff) needs to be higher than the highest frequency content in the physiologic signal but not so high as to allow unnoticed high-frequency noise to be acquired. High-frequency noise can cause software algorithms to trigger on the noise instead of the physiological waveform and calculate incorrect results. If a sensor has a bandwidth that is very high with respect to the frequency content of the signal, it is imperative that the signal be subjected to a low pass filter to lower the bandwidth to an optimum value before being acquired by the computer system. The old saying holds that more is not always better.

Biopotential

Important physiological signals that can be measured as biopotentials include the electrocardiogram (ECG or EKG), electroencephalogram (EEG), electromyogram (EMG), electrooculogram (EOG) and nerve activity, typically sympathetic nerve activity (SNA). Some implantable telemetry devices allow
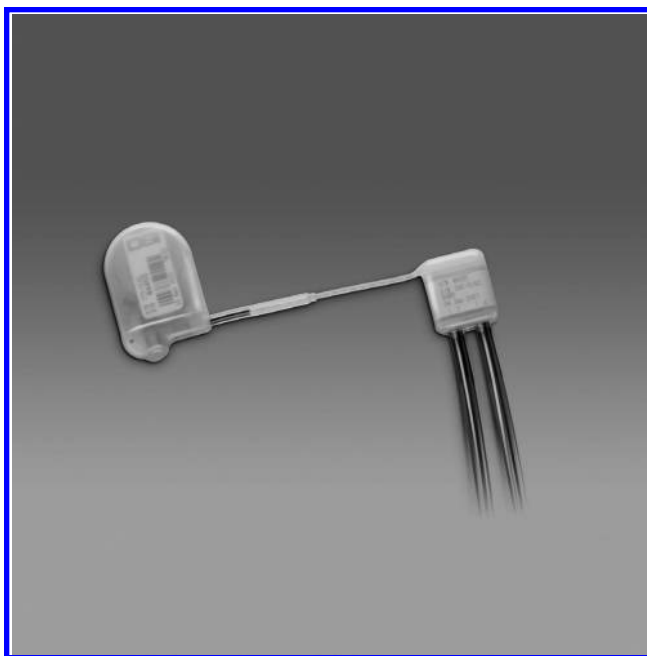
Figure 13.5 CNS telemetry: Some transmitters are uniquely configured for CNS needs. Pictured is a transmitter with four biopotential lead pairs and a separate battery pack. This configuration allows the biopotential leads to remain in place for long-term monitoring while the power source is replaced. Multiple biopotential leads allow for flexible configuration of any combination of EEG, EMG, EOG or ECG recording.

multiple biopotential signals of the same or varying types to be monitored at the same time (see Figure 13.5).

Biopotentials are simply electrical voltages that are measured by a voltmeter. Initially these were called galvanometers, such as the one invented by Willem Einthoven, who was awarded the Nobel Prize in 1924 for developing the electrocardiograph. Although modern instruments for the measurement of biopotentials are much more sophisticated and have been dramatically miniaturized to allow implantation, the basic principle of operation has not changed significantly since Einthoven's day.

Given that the actual biopotentials that are created by physiologic organ systems such as the heart are so small, high-quality, noise-free signals are very difficult to achieve with electrocardiographs that are plugged into an alternating current power source such as a wall outlet. Since the line voltage is so high (120 or 220 V) compared to the tiny millivolt signals being measured, the line noise from the power source often obscures the signal. The use of telemetry is ideal for this application since the device is powered by an onboard direct current battery and is therefore immune from the noise artifacts common in the hardwired instruments.

Important characteristics of biopotential sensors include signal bandwidth (often referred to as frequency response), the amplitude of signals they are designed to measure and the input impedance of the sensor relative to the electrodes.

Temperature

Temperature was the first, and is the simplest, physiological parameter to have been monitored from experimental animals via telemetry. Most implantable telemetry devices provide a measure
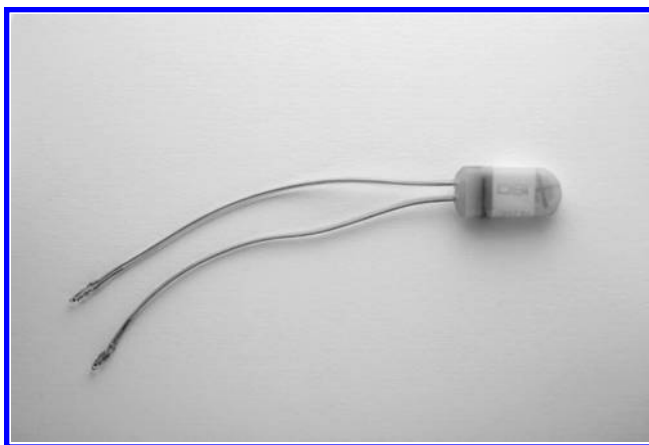
Figure 13.6 Temperature sensing transmitter: The pictured transmitter is equipped with two separate temperature probes. Each probe can be placed in different locations and will transmit two unique temperatures simultaneously.

of temperature by the use of a thermistor. The word *thermistor*, derived by combining the words *thermal* and *resistor*, is a specialized type of resistor whose resistance varies significantly with temperature. By passing a fixed voltage through the thermistor, the current can be measured and resistance thereby calculated. Through a process of calibration, the resistance can be accurately and precisely converted into a temperature value (degrees Celsius or Fahrenheit).

Thermistors used in implantable telemetry are either located on the tip of a probe connected by wires to the implant body or by incorporating the thermistor inside the body itself. Most commonly, the implant is placed well inside the animal's body, thus providing a measure of core body temperature. Investigators should be cautious in interpreting core temperature data from implants that are placed subcutaneously since the proximity to the animal's external environment may result in measurements that differ considerably from true body core temperature.

Thermistors located at the end of a probe are typically used for precise temperature measurements in a small region of the body (see Figure 13.6). Brown fat deposits in metabolism/energetics research are examples of this application.

Motor Activity

Motor activity is an important parameter in the assessment of drug effects on the central nervous system (CNS). An increase in spontaneous motor activity can be caused by excitation and a decrease can be an indication of sedation. Motor activity is specified as a part of the Core Battery for drug safety assessment in the ICH Topic S7A regulatory guideline.

Historically a general indication of motor activity from telemetry has been derived from changes in received signal strength as an animal moves around in its cage. The signal strength changes as the distance between the implanted animal and the receiver varies. This method has proven adequate for detecting relative increases and decreases in motor activity, but is not quantitative.

Newer implantable telemetry devices used in large animals provide a quantitative and much more sophisticated measure of motor activity through the use of three-axis accelerometers inside the implant. These are the same accelerometer devices that have become common in smart phones and video game controllers. Changes in an animal's position and motion can now be very precisely detected and measured with these systems using appropriate software algorithms.

Surgical Implantation Techniques

Telemetry devices are implanted using aseptic surgical techniques appropriate to all clinical and nonclinical surgeries. A variety of surgical techniques are required to allow for the use of telemetry with the different species and variety of applications used in nonclinical drug safety assessment. To obtain systemic arterial pressure data, the pressure sensor must be placed within freely flowing blood, often in the animal's aorta. The insertion site can vary greatly with species (e.g. carotid artery in the mouse, aorta in the rat, femoral artery, iliac artery or aorta in large laboratory animals, to name only a few).

For the collection of electrocardiograms (ECGs), a pair of biopotential leads (one positive and one negative) are typically placed on shaved skin with adhesive electrodes, underneath the skin (subcutaneously), or in a small intramuscular pocket in an assortment of configurations. They may also be placed directly on the epicardium of the heart, which minimizes interference from muscular activity. Technically, this is referred to as a "cardiac electrogram" instead of an electrocardiogram. The recent development of intravenously placed negative biopotential leads with implantable telemetry devices allows for signal collection with negligible interference from animal activity, similar to epicardial electrodes, but does not require the invasiveness of a thoracotomy surgery.

For collection of electroencephalography (EEG) data, small holes are created in the skull to allow for epidural lead placement. Researchers may also use biopotential leads with telemetry devices to measure electrical potentials in a variety of other applications (electromyogram, electrooculogram, etc).

Surgical training and proficiency is critical to ensuring that telemetry devices are appropriately placed to deliver high-quality data with optimal longevity while ensuring animal welfare. Appropriate surgical training is required by government agencies regulating the use of animals in research, as well as by funding sources, scientific publishers and institutional animal care and use committees. Regulations vary by country; in some locations only veterinarians are allowed to perform surgery on research animals, while in others (e.g. the United States) veterinary technicians and other specially trained personnel may do so as well. Nonveterinarian "research surgeons" can achieve voluntary certification of their proficiency through the Academy of Surgical Research (ASR). Educational and training opportunities may also be provided by other organizations such as the American Association of Laboratory Animal Science (AALAS), by telemetry or animal vendors, by individual veterinarians or surgeons with extensive experience, or by other specialty training groups. Animals may also be purchased with the telemetry devices already implanted from a variety of sources.

EXPERIMENTAL DESIGNS FOR TELEMETRY STUDIES

There are several benefits of collecting *in vivo* physiology data from conscious, unrestrained animals by telemetry. Unlike anesthetized animal models, the animals' neurohumoral regulatory systems are intact and, unlike physically restrained animals, the animals are in a more relaxed and responsive physiologic state (low sympathetic nervous system activation). However, perhaps the most important advantage is that the animals can be studied in crossover experimental designs, such as the Latin square, where statistical power (sensitivity) to detect acute changes is increased and the number of animals required is dramatically reduced.

A common cardiovascular study design in pharmaceutical safety assessment of small molecule drugs that utilizes telemetry is the 4 × 4 Latin square with four animals, four treatment groups (control, low, mid, and high dose) and four different treatment days (see Table 13.2). In this balanced study design, all four animals receive all four treatments, but in different orders. Also, all four treatments are equally represented on each treatment day so that environmental differences

Table 13.2   Animals and Treatments in a Simple 4 × 4 Latin Square Experimental Design

	N = 4			
Animal ID	Period 1 (Day 1)	Period 2 (Day 4)	Period 3 (Day 8)	Period 4 (Day 11)
Animal 1	C	L	M	H
Animal 2	L	M	H	C
Animal 3	M	H	C	L
Animal 4	H	C	L	M

do not preferentially affect a single treatment group. Because the same animals are studied for all treatment groups, the variation between animals can be removed from the error term of the analysis of variance (ANOVA), thus making this a more powerful design.

Contrast the 4 × 4 Latin square design with the parallel design used in traditional toxicology studies (Table 13.3). Note that 16 animals are required and each animal is permanently assigned to its treatment group. The variability among animals is distributed (or confounded) with the treatment effects, thus reducing the statistical power compared to the Latin square design. This limitation can be partially offset by dramatically increasing the number of animals used in the study.

A significant limitation of the 4 × 4 Latin square design depicted in Table 13.2 is that it's sensitivity is dramatically affected by any missing data (In statistical terms, this is because there are very few degrees of freedom in the error term). Missing data can occur due to failure of instrumentation or the loss of an animal due to health issues before the entire dosing schedule is complete. A more powerful approach, which is much less vulnerable to missing data, is to execute a double 4 × 4 Latin square design, such as is depicted in Table 13.4. The use of a double Latin square design can provide the benefit of increased sensitivity to detect or rule out drug effects, even if animals are lost from the study, thus reducing the risk of having to repeat a study. Given that safety pharmacology telemetry animals are typically maintained in a colony for reuse on several studies over time (as much as 2 years), these benefits are obtained without incurring a substantial increase in the total number of animals consumed.

Although the Latin square crossover designs described above are very powerful for detecting acute pharmacological effects, they are impractical for evaluating chronic effects such as those caused by large molecule drugs or effects that occur in repeat dose safety assessment studies. In response to

Table 13.3   Animals and Treatments in a Parallel Experimental Design

Animal	N = 16	Dose
1–4		C
5–8		L
9–12		M
13–16		H

Table 13.4   Animals and Treatments in a Double 4 × 4 Latin Square Experimental Design

	Double 4 × 4 Latin Square Design N = 8			
Animal ID	Period 1 (Day 1)	Period 2 (Day 4)	Period 3 (Day 8)	Period 4 (Day 11)
Animals 1 and 5	C	L	M	H
Animals 2 and 6	L	M	H	C
Animals 3 and 7	M	H	C	L
Animals 4 and 8	H	C	L	M

recommendations developed by the ILSI Health and Environmental Sciences Institute (HESI) in 2011, the collection of functional cardiovascular safety endpoints such as ECGs and blood pressure from traditional nonrodent repeat dose toxicology studies using external telemetry has become increasingly common. Although the sensitivity to detect small changes is limited compared to the acute Latin square designs because of the parallel experimental design and confounding effects of other activities that occur with toxicology studies, technology, methods, and study procedures have improved considerably in recent years and moderate chronic cardiovascular functional effects can now be detected.

CONCLUSION

The development of telemetry for the remote monitoring of physiological signals from laboratory animals has dramatically improved the nonclinical safety assessment of human drug candidates. Prior to the 1990s, effects of drug candidates on functional endpoints such as blood pressure, heart rate, the electrocardiogram, and cardiac function were very crudely assessed, if they were assessed at all, in physically or chemically restrained animals in toxicology studies. This resulted in several drugs advancing into clinical development with adverse pharmacological effects, most notably proarrhythmic effects due to electrocardiographic QT interval prolongation.

With the advent of nonclinical drug safety assessment studies known as Safety Pharmacology in the late 1990s, monitoring of cardiovascular safety by implanted and/or external telemetry became commonplace. Subsequently, these methods have been applied to standard repeat dose nonrodent toxicology studies, primarily for biotechnology-derived drug candidates.

In order to ensure that drug safety data derived through telemetry are accurate and useful, it is critical that scientists, technical staff, surgeons, and test facility management have an understanding of the strengths, weaknesses, and proper application of telemetry systems, appropriate for their role in the implementation of the technology and the conduct of the studies.

This chapter has provided an overview of the various factors to be understood to properly implement telemetry in a drug safety assessment facility and ensure that sponsors and regulatory agencies can make well-informed decisions on the safety of drugs as they advance through the drug development process. Further detail can be obtained from the section "Suggested Readings" at the end of the chapter.

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Molecular Imaging Techniques in Laboratory Animals

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CONTENTS

INTRODUCTION

Drug development is a lengthy, high-risk, and costly process, which usually spans 10–15 years following the established paradigms.[1] Drug companies currently have hundreds of thousands of potential drug compounds; however, using traditional discovery and development methods, less than 1 of every 10,000 will eventually enter the market as an FDA-approved treatment. The timeline and success rate of drug development can be improved by the use of highly specific target related

to abnormal or narrowly defined genetic mutation related to orphan disease indication in laboratory animal models. The most recent technique available for real-time molecular imaging of the disposition of drug in living body provides a unique, early opportunity to identify whether a drug candidate hit the target or not in laboratory animal at the early stages of drug development, thereby improving the quality of the molecules ultimately selected to move forward. This cutting-edge research area is so important that a separate chapter is dedicated to application of imaging techniques in laboratory animals for preclinical drug development process.

Molecular imaging technology is the visualization, characterization, and measurement of biological processes at the molecular and cellular levels in humans and other living systems. In the past decade, molecular imaging technology is increasingly recognized as an important factor of preclinical and clinical research tool to speed up the long-timelines of drug development process.[2] This emerging field focuses on using non-invasive techniques to visualize the anatomic structures and physiologic activity in animal model in preclinical research and patients in clinical practice. Modern imaging assessments provide information about tissues by penetrating the living body via physical phenomena (see Figure 14.1), such as magnetic field/radiofrequency (magnetic resonance imaging/MRI), x-ray (computed tomography/CT), high frequency sound waves (ultrasound/US), optical (bioluminescence/fluorescence), gamma rays (single photon emission computed tomography/SPECT), and annihilation twin photons from beta emission (positron-emission tomography/PET). These modern imaging modalities have been re-engineered for use with laboratory animals by pushing the resolution and sensitivity of each modality to the physical limit. The improved high resolution and extreme sensitivity make the imaging technology highly translational in the preclinical drug development process.
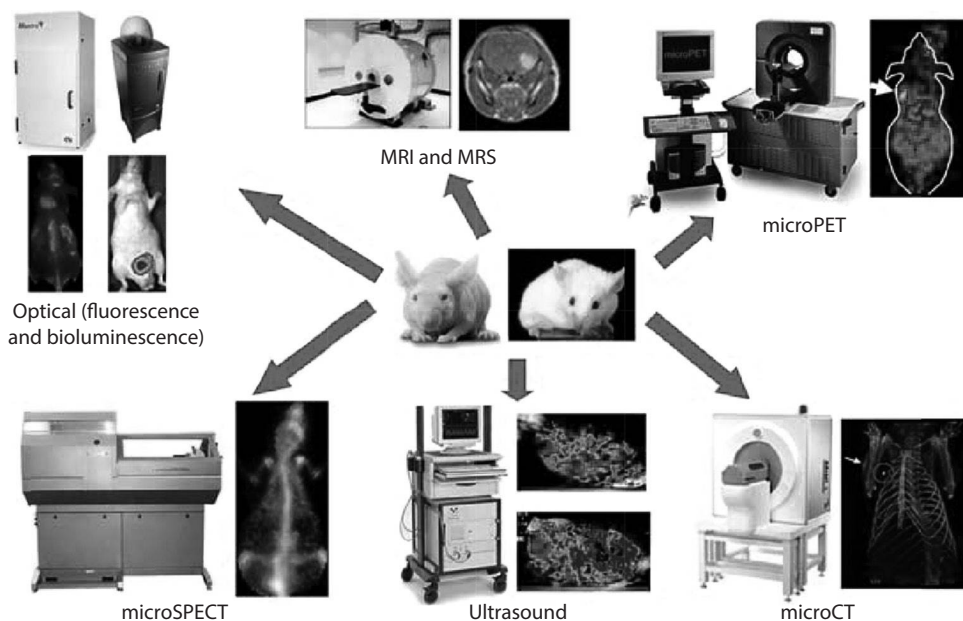
Figure 14.1 Multimodality imaging instrumentations. The modern molecular imaging equipment includes magnetic field/radiofrequency (magnetic resonance imaging/MRI), x-ray (computed tomography/CT), high frequency sound waves (ultrasound/US), optical (bioluminescence/fluorescence), gamma rays (single photon emission computed tomography/SPECT), and annihilation twin photons from beta emission (positron-emission tomography/PET). (Images provided by LoMIN of NIBIB at National Institutes of Health, Bethesda, MD.)

The cutting edge non-invasive imaging technology is able to provide evidence of in vivo biodistribution of imaging probes, confirm on-target biological activity, illustrate disease mechanism, and validate efficacy of drug treatment. Maximizing collection of in vivo biological information during the preclinical development can be highly valuable during the critical period of lead selection/optimization of promising drug candidates. The use of molecular imaging at these stages can provide critical information that was previously unavailable or available only indirectly through other interpretations in long-term preclinical studies. The use of molecular imaging in preclinical development, when balanced with well-established and accepted practices, can significantly reduce both time and expense in the development and commercialization of drug candidates including both small molecules and biologics. Several excellent review articles have been published on the general role of molecular imaging on the process of both preclinical and clinical drug development.[3,4] In this book chapter, we will mainly focus on exploring the role of molecular imaging techniques in preclinical development in laboratory animal models of human diseases and novel solutions to improve the efficiency of the whole spectrum of the drug development process.

MULTIMODALITY IMAGING TECHNIQUES

MRI, SPECT, PET, CT, Ultrasound, and optical imaging all generate sectional images that can be reconstructed into two-dimensional (2-D) or three-dimensional (3-D) images. For preclinical evaluations, the imaging technique must have spatial resolution that is adequate for small animals and sensitivity to detect biochemical events. Each technique has certain advantages and limitations; platform selection is dependent upon which techniques can provide the greatest complementary information toward answering a particular preclinical question. Combining multimodality imaging techniques in the same animal, such as PET/CT, SPECT/CT, and PET/MRI co-registration, can provide temporal pathophysiological information such as development of structural and functional changes, progression and resolution during disease treatment, and the drug-related on target and even off target related toxicities.

Imaging Probes and Biomarkers

Molecular imaging (MI) probes are agents used to visualize, characterize, and measure biological processes in living systems. Molecular imaging instrumentation, including MRI, CT, US, optical, SPECT, and PET as shown in Figure 14.1, are tools that enable the visualization and quantification in space and over time of signals from MI probes. In preclinical research, diagnostic probes are administered in trace amounts and typically do not induce any physiological response or significant pharmaceutical effect in living systems.

Thousands of imaging probes, including radiolabeled small molecules, biologics, nanoparticles, and different cell types, have been developed and tested in preclinical research in laboratory animal models and some of them used in routine clinical practice.[5] A radiotracer can be defined as a specific radiolabeled molecule that monitors the in vivo behavior of a functional molecule and can be used to provide biological information in a living system. The synthesis of a radiotracer generally includes radiolabeling of test article, purification of final product, testing specific activity and analysis of in vivo stability. The commercially available imaging probes and customized radiotracers for PET or SPECT imaging can be used to detect such functional changes as cerebral blood flow, myocardial perfusion, glucose metabolism, infection, bone lesion, kidney function, apoptosis, angiogenesis, thyroid dysfunction, tumor detection, gene expression, neuroreceptor binding, and cell trafficking process.[6]

FUNCTIONAL MOLECULAR IMAGING TECHNIQUES

The radiotracer approach used for PET and SPECT functional imaging is especially valuable at early stages of drug discovery, where researchers can directly label a drug candidate to see where it localizes in laboratory animals. If the drug candidate does not distribute to its intended physiologic target, as desired, it can be excluded from further development at an early stage. In addition, PET and SPECT imaging experiments can reveal molecular changes in targeted and non-targeted tissues brought about by the drug and its side effect caused within the laboratory animals. This is an extremely important capability in view of the fact that changes on the molecular level occur long before becoming visible in any anatomic imaging. Such approaches allow the rank ordering of development candidates based on relevant physiologic processes very early in drug development.

In vivo imaging techniques such as PET and SPECT provide a means to perform pharmacokinetics (PK) and pharmacodynamics (PD) studies in animal models without sacrificing the animal. With the introduction of dynamic and static imaging approaches, it is now possible to obtain high-resolution PET and SPECT images of "motion-frozen" time points in PK and PD studies and be able to quantify the amount of drug in the target organs of interest in the same laboratory animal over a period of time during the drug evaluation process.

SINGLE-PHOTON EMISSION COMPUTED TOMOGRAPHY (SPECT)

SPECT camera images individual high-energy photons—gamma rays, resulting from radionuclide decay. The technique requires injection of a gamma-emitting radioisotope into laboratory animal for research or patient in clinical practice. Radioisotopes can be coordinated to a test article (ligand) to allow for the evaluation of the binding properties of the test article *in vivo*. The combination of radioisotope and the ligand will bind to a place of interest in the body of the living subject to be seen by the gamma ray detectors, thus producing an image by computed tomography. For example, an imaging agent with affinity for areas of growing bone can be utilized to evaluate potential impact of a test article on the process of ossification (see Figure 14.2).

The single-photon emission radiates from the source in all directions with equal probability. Because of this, an aperture composed of highly attenuation material is required to identify the path of origin of each emission prior to being able to create an image with a gamma camera. In preclinical SPECT imaging, the most recently developed multi-pinhole collimator technology achieves submillimeter, high resolution in small laboratory animals.

SPECT can be used to complement any gamma imaging studies. Biodistribution, tumor imaging, infection, bone imaging, and cardiac gated imaging can also be performed with SPECT.[7] SPECT offers radiolabeled image resolution and sensitivity that can translate from mouse to human. It also can provide both qualitative and quantitative measurement of physiologic processes. The isotopes used for SPECT imaging (i.e.,[123]I, ^{125}I, ^{99m}Tc, ^{111}In) are readily available and relatively inexpensive, with longer half-lives than those commonly used in PET imaging. The extended half-life of these isotopes translates to longer scan times and/or scanning periods without the need for secondary isotope administration. These characteristics of SPECT imaging results in a reduction in the isotope quantity needed, as well as, the total number of animals utilized in preclinical studies—more data using fewer animals over a longer investigational period. SPECT imaging/isotopes also provide the ability to simultaneously image multiple radiopharmaceuticals with different energies (i.e., ^{99m}Tc vs. [125]I). MicroSPECT functional imaging is able to co-register with a high-quality CT imaging framework for anatomical imaging comparison (see Figure 14.3).

One characteristic of SPECT imaging is the infrastructure and instrumentation cost, as well as, the need for radioactive material handling and dosimetry. This platform requires the investigators'
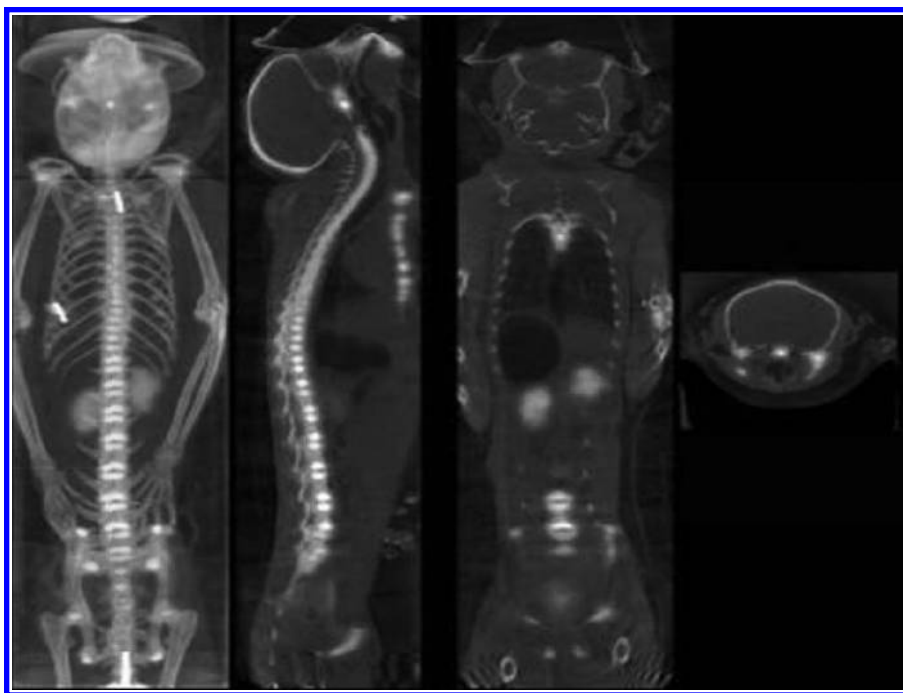
Figure 14.2 SPECT/CT images of NHP. This collection of images demonstrates SPECT/CT imaging of a non-human primate model (*simia sciureus,* Squirrel Monkey). Isotope/Imaging parameters: 3 mCi of [99]mTc-MDP; dose administration 75 min prior to SPECT scan initiation; SPECT scan duration of 56 min; CT scan duration of 25 min. (Image analysis provided by MPI Research Inc., Mattawan, MI and inviCRO LLC, Boston, MA.)

multidisciplinary knowledge and experience to ensure the study design provides an adequate data set to meet their objectives. Although the molecular sensitivity of SPECT is almost one order of magnitude lower in sensitivity than PET, SPECT imaging continues to be widely used in both clinical practice and preclinical research due to its advantages of a relatively lower operating cost, dual-labeled compounds, and a valuable long half-life radionuclides, which allow for lengthier monitoring periods of in vivo biological process compared to PET imaging.

Positron-Emission Tomography (PET)

In PET imaging, a compound (small-molecule drug or biological molecule) labeled with a positron-emitting radioisotope is injected into the subject in a non-pharmacological trace quantity. A positron ejected by a radionuclide combines with an electron in adjacent tissue to emit a pair of photons (511 keV) resulting from annihilation of a positron-electron pair. The PET scanner uses the annihilation coincidence detection (ACD) method to obtain projection images of the localization and quantification of the radiolabeled compound in the living subject.[8] PET imaging technology can be used for drug distribution,[9] organ perfusion, cell trafficking,[10] tumor targeting, tumor metabolism/proliferation, tumor angiogenesis (see Figure 14.4), tumor hypoxia, tumor apoptosis, tumor volume, anti-cancer therapeutic response,[11] bone growth/healing, and mechanism studies in animal models of CNS and autoimmune diseases.[12,13] Advantage of this technique includes high molecular sensitivity (nanomolar) with almost unlimited depth penetration. Disadvantages of PET include radiation safety and relatively high cost. PET functional imaging is often combined with either micro CT or animal MRI for better identification of functional change in deep tissue against anatomical structure in an in vivo animal model.
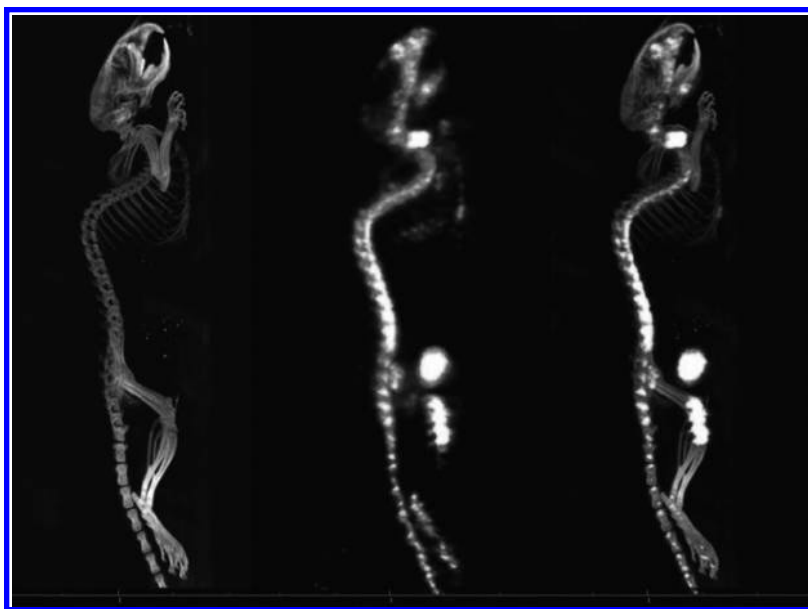
Figure 14.3 Micro SPECT/CT imaging of mouse injected with ^{99m}Tc-MDP for monitoring osteoblastic activity. (a) Micro CT image offers good anatomical information. (b) Micro SPECT image offers functional or physiological information. In this case ^{99m}Tc-MDP was used to visualize osteoblastic activity. (c) Micro CT/SPECT fused imaging can offer additional insights by providing anatomical locality to sometimes cryptic physiological data. (Images provided by Van Andel Research Institute, Grand Rapids, MI.)

1 h   4 h   20 h   44 h
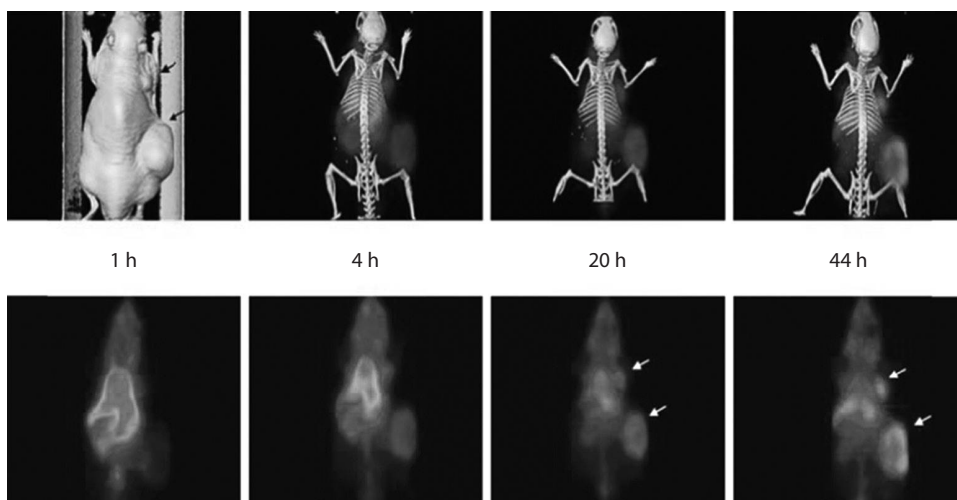
Figure 14.4 PET/CT images of tumor angiogenesis. This figure shows microPET/CT fused images acquired at 1, 4, 20, and 44 h post-injection of 200 μCi/40 μg ^{64}Cu-bevacizumab in a MIAPaCa-2 pancreatic xenograft tumor model. The top-left sub-figure contains either surface rendering image performed using AmiraTM (Mercury Computer System Inc.) to show relative tumor location (arrows). The dynamic imaging data show whole-body distribution of ^{64}Cu-bevacizumab in tumor animal model in which the tumor sites are pointed to by arrows. (Images provided by MPI Research Inc., Mattawan, MI; Van Andel Research Institute, Grand Rapids, MI and UTHSCSA, San Antonio, TX.)

Micro X-Ray Computed Tomography (CT)

Computed tomography is an application of x-ray imaging that provides a three dimensional anatomical image.[14] In CT scan, x-rays are emitted from an x-ray source rotating around the subject placed in the center of the CT scanner. A detector opposite the x-ray source senses x-rays that are not absorbed by the tissue; this absorption is inversely related to the density of the tissue structures. The x-ray absorption profile is then used to reconstruct high-resolution (roughly 6–50 μm with no depth limit) tomographic anatomical images. MicroCT can be used for bone studies (e.g. osteoporosis, see Figure 14.5), developmental and reproductive toxicology (DART) study (see Figure 14.6),
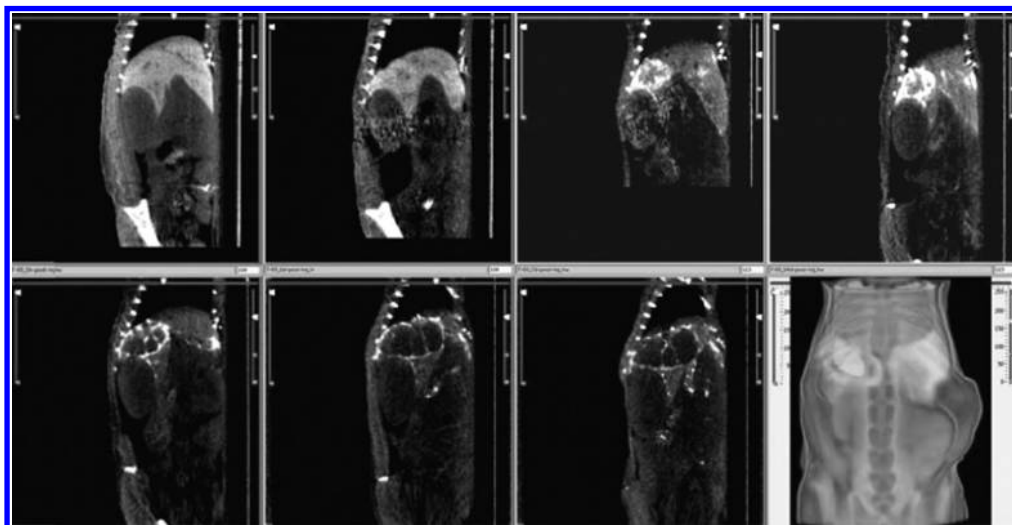
Figure 14.5 Typical whole-mouse microCT images of tumor model. The images were reconstructed from a tomographic data set as 2-dimensional sagittal slices (black and white) showing bone structure and abdominal soft tissues. The microCT surface image (bright white and surrounding grey here) is also shown for appreciation of the tumor size on the surface of the flank position. (Images provided by Van Andel Research Institute, Grand Rapids, MI.)
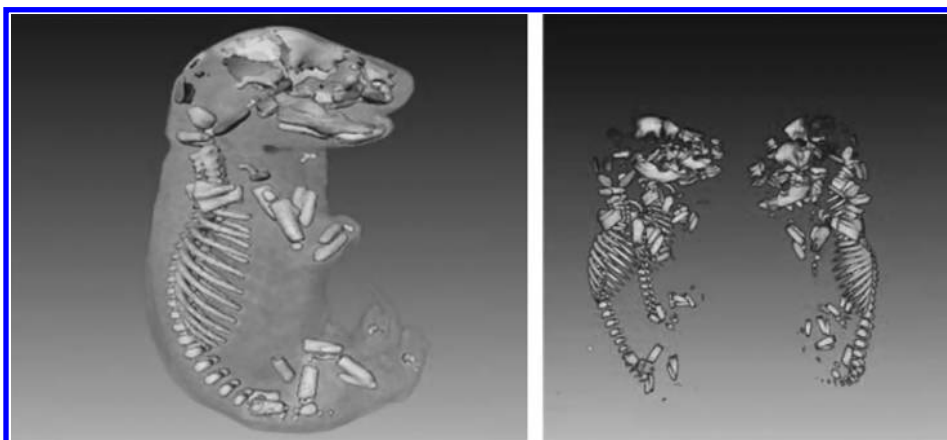
Figure 14.6 CT Imaging of Developmental and Reproductive Toxicology (DART). The images were reconstructed from a tomographic data set as 360 projection showing bone structural development. CT scan duration of 25 min. (Image reconstruction provided by MPI Research Inc., Mattawan, MI.)

(a)   (b)

(c)
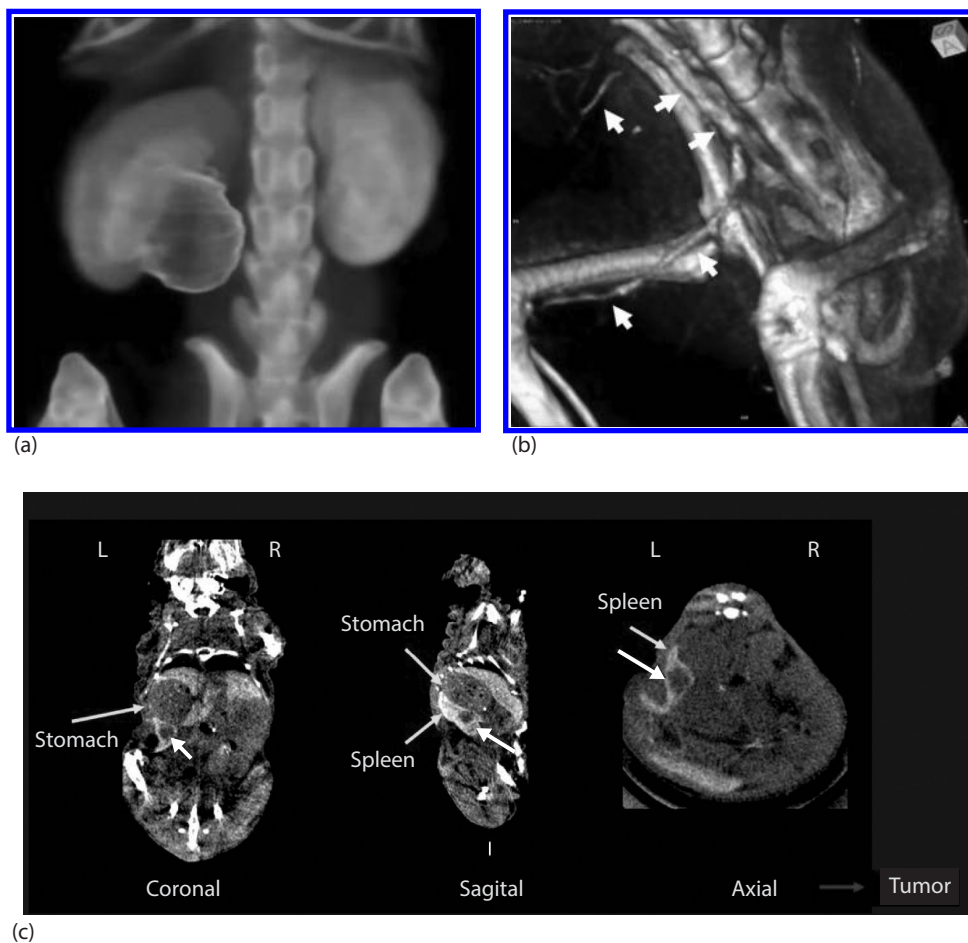
Figure 14.7 CT images of kidney function. (a) Image of a kidney cyst transgenic mouse model after 3D reconstruction. A cyst in the right kidney has been clearly revealed through micro CT images of a mouse injected with contrast agent. (b) Image of vascular structure (arrows) in a healthy mouse after 3D reconstruction. Vascular structure can be identified by micro CT images when mice are injected with appropriate contrast agent. (c) Images of a pancreatic tumor liver metastasis orthotopic mouse model. Mice with appropriate contrast agent scanned by Micro CT can be used to identify the tumors or pathological lesions. The contrast of liver lobes and spleen had been increased by injecting liver CT contrast agent into mouse. The dark area pointed by arrows in the spleen is a metastatic tumor. (Images provided by Van Andel Research Institute, Grand Rapids, MI.)

fetal skeletal evaluations vascular studies, and lung studies. The use of contrast media enables soft tissue segmentation and some functional imaging, for example, in kidney function studies (see Figure 14.7). The disadvantages of microCT include low soft-tissue contrast, use of radiation, and limited molecular-level applications. MicroCT is able to provide a high-quality anatomical framework for functional imaging techniques, particularly in PET and SPECT imaging data analysis.

Magnetic Resonance Imaging (MRI)/Magnetic Resonance Microscopy (MRM)

MRI utilizes nuclear magnetic resonance (NMR) and the signal is derived primarily from the hydrogen nuclei (protons) of water molecules. The technique uses a powerful magnetic field to
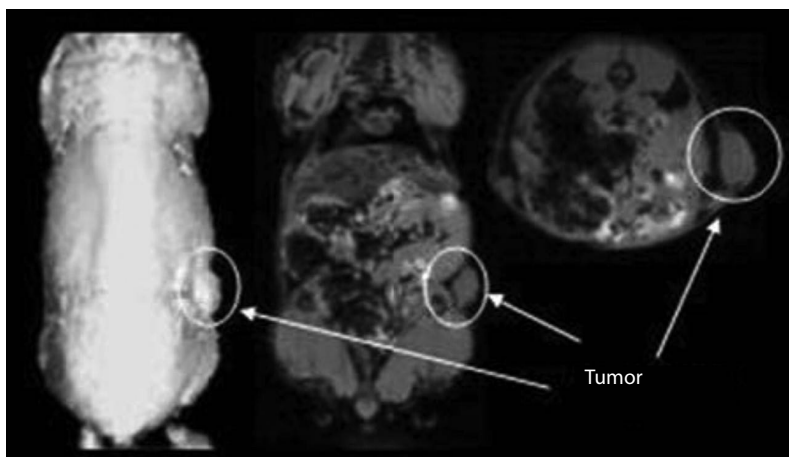
Figure 14.8   MRI imaging of xenograft pancreatic cancer mouse model. MRI scan was performed in a xenograft mouse tumor model with a tumor size (~250 mm^3) at day 16 after tumor implantation with UKPAN-1 cancer cells. The image demonstrates the tumor located at the right flank. (Images provided by UTHSCSA, San Antonio, TX.)

align the magnetization of atoms in the organism and a pulse of radio frequency to alter the alignment of this magnetization. The scanner then detects the rotating magnetic field to produce an image of the scanned area.[15] Unlike radiography or CT, no ionizing radiation is used. Intravenous contrast agents are used to enhance the signal and/or help delineate vessels or tumors. MRI is most useful for imaging soft tissues, especially those with little density contrast, such as the liver or brain, and is most frequently used to provide anatomical images and delineate lesions such as tumors or areas of necrosis in living animal models (see Figure 14.8). Magnetic resonance microscopy (MRM) is magnetic resonance imaging with resolutions of better than 100 μm^3. Advantage of this technique is high resolution (roughly 10–100 μm with no limit of depth) and high soft-tissue contrast. The disadvantage includes limited molecular applications and long scanning times. Functional information can be gathered in a related technique known as magnetic resonance spectroscopy (MRS),[16,17] which provides information either on the concentration and distribution of magnetic nuclear isotope-labeled drugs in tissues of laboratory animal or on particular endogenous biochemical (metabolites) since a specific pattern of metabolites can be associated with certain diseases and tumors.

OPTICAL IMAGING

A variety of different kinds of optical imaging techniques have been developed for biomedical applications. They include various microscopy methods such as confocal microscopy, two-photon microscopy, and coherent anti-Stokes Raman scattering (CARS) microscopy for in vitro and ex vivo applications. These techniques are combined with a number of methods for in vivo applications such as various types of microscopic imaging, bioluminescence imaging, fluorescence imaging, diffused optical tomography, and optical coherence tomography to name a few. Over the last decade, the various modes of optical imaging have grown in popularity and sophistication. Today optical imaging has a wide variety of applications in genomics, proteomics, cell biology, and drug discovery and development. Optical imaging has emerged as a real-time, sensitive, and non-invasive modality for visualization, localization, and measurement of bioactive molecules and molecular processes *in vivo.*

Even though optical imaging still cannot compete with PET, SPECT, MRI, and CT in clinical applications today, the advantages of optical imaging (i.e. convenience, sensitivity, cost effectiveness, and non-radioactive material safety) have made it very popular among the traditional imaging modalities for molecular imaging in preclinical studies. Optical imaging of receptors, enzymes, gene expression, live cells, and tumors in vitro and in vivo has deepened our understanding of disease progression and therapeutic response at the molecular, cell, tissue, and whole-animal levels.

In vivo optical imaging includes fluorescence and bioluminescence imaging. Both techniques are highly sensitive (picomolar) at limited depths of a few millimeters, quick and easy to perform (with a high-throughput capability), and in general do not require costly instrumentation.[18] This character makes it particularly suited to the drug development and validation process.

Fluorescence imaging uses high intensity illumination of a certain wavelength to excite fluorescent molecules in a sample. When a molecule absorbs photons at the appropriate wavelength, electrons are excited to a higher energy level. As these excited electrons "relax" back to the ground-state, vibrational energy is lost and, as a result, the emission spectrum is shifted to longer wavelengths. Fluorescence imaging causes excitation of certain fluorophores in a living system by using external light and then detects fluorescence emission with a sensitive CCD camera. The fluorophores can be endogenous molecules (such as collagen or hemoglobin); exogenous fluorescent molecules such as green fluorescent protein (GFP) or small synthetic optical contrast agents (see Figure 14.9).

A limitation of in vivo fluorescence imaging is from light attenuation and scattering by adjacent living tissues. Light in the near infrared (NIR) range (650–900 nm) instead of visible light can improve the light penetration within laboratory animal. It can also minimize the auto-fluorescence of some endogenous molecules such as hemoglobin, water, lipids and other biomacromolecules.

(a)　　　(b)
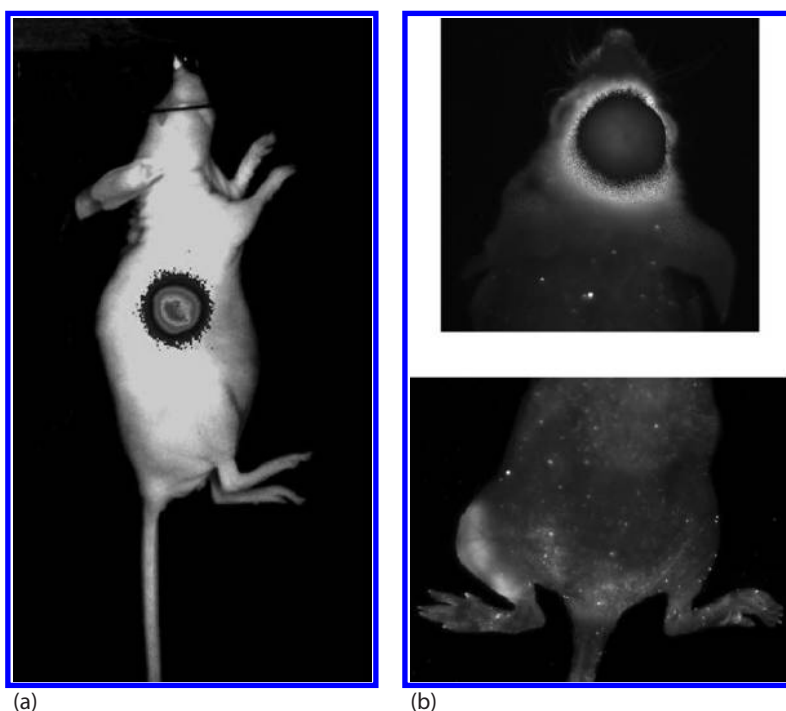
Figure 14.9 In vivo optical imaging images. (a) Bioluminescence image (BLI) of a mouse glioblastoma xenograft model. (b) Fluorescence image of a mouse glioblastoma orthotopic model. (c) Fluorescence image of a mouse osteosarcoma orthotopic model. Tumor cells express GFP. (Images provided by Van Andel Research Institute, Grand Rapids, MI.)

In a whole mouse illumination experiment, photon counts in the NIR range (670 nm) are about four orders of magnitude higher compared to those in the green light range (530 nm) under similar conditions. Near-infrared fluorescence imaging has provided an effective solution for improving the imaging depth along with sensitivity and specificity. Therefore, fluorophores with emission maxima in the NIR region are important for successful in vivo optical imaging and future clinical applications. Commercially available fluorophores, with well-defined excitation and emission spectra, can be used to "stain" specific structures or molecules in a specimen. Judicious choice of fluorophores allows the identification of multiple targets as long as emission spectra can be cleanly separated and distinguished from auto-fluorescence.

Ultrasonography (US)

The principles of ultrasound imaging are founded in the basic physical interaction of sound waves with living tissues of various densities. Ultimately the results of these interactions are displayed as an image. Doppler-based modes can also be utilized to evaluate fluid dynamics, primarily velocity, of the circulatory system or within specific organs. Ultrasonography can be used to evaluate both structural and functional endpoints of multiple target organs within various species, including small and large animals. The utilization of ultrasound imaging in research projects has become a key tool due to the noninvasive nature of the technology, as well as, the advancement of the technology to provide greater imaging resolution in very specific settings. Contrast agents, such as microbubble, can be functionalized with specific molecules, such as monoclonal antibody, thus improving image quality.[19] Micro ultrasound is a useful tool for volume measurement. It is more precise and accurate than conventional methods which use calipers for measurement (see Figure 14.10).

Ultrasonography is practiced in various formats such as brightness mode (B-mode), motion mode (M-mode), Doppler (including both Color Flow and spectral Doppler) and contrast-enhanced imaging. A distinct advantage to the utilization of ultrasonography is simply the many applications that can be practiced in preclinical development. Subjects can be imaged at multiple timepoints within a given study, and multiple targets can be evaluated. A disadvantage is the lack of high-resolution capabilities, as well as, the need for an experienced imaging expert to obtain the acceptable images for analysis. Poor image capture will ultimately lead to unsatisfactory data sets. Ultrasonography represents a noninvasive imaging solution that can be utilized to provide quantitative structural and functional data sets. Investigators have many options available through the application of ultrasonography to match the imaging format to the specific needs of their research projects.

APPLICATIONS OF PRECLINICAL IMAGING IN LABORATORY ANIMALS

Molecular Imaging as an ADME Platform in Drug Screen

Greater than 80% of investigational new drugs fail during drug development because of unsatisfactory absorption, distribution, metabolism, and excretion (ADME) characteristics. Tools that help to predict the ADME responses early in development present an advantage as they avoid wasting valuable resources on compounds that will to fail. A multitude of non-invasive, high-resolution, imaging technologies have developed regular uses in the study of ADME processes during drug discovery and development. In this section, we will overview PET, SPECT, QWBA, and other techniques, emphasizing the importance of these technologies in ADME platform for drug discovery and drug development.

Traditional preclinical studies on absorption, distribution, metabolism and excretion, or ADME studies are currently conducted by introducing a radiolabeled drug candidate into laboratory

(a)

(b)

(c)

(d)
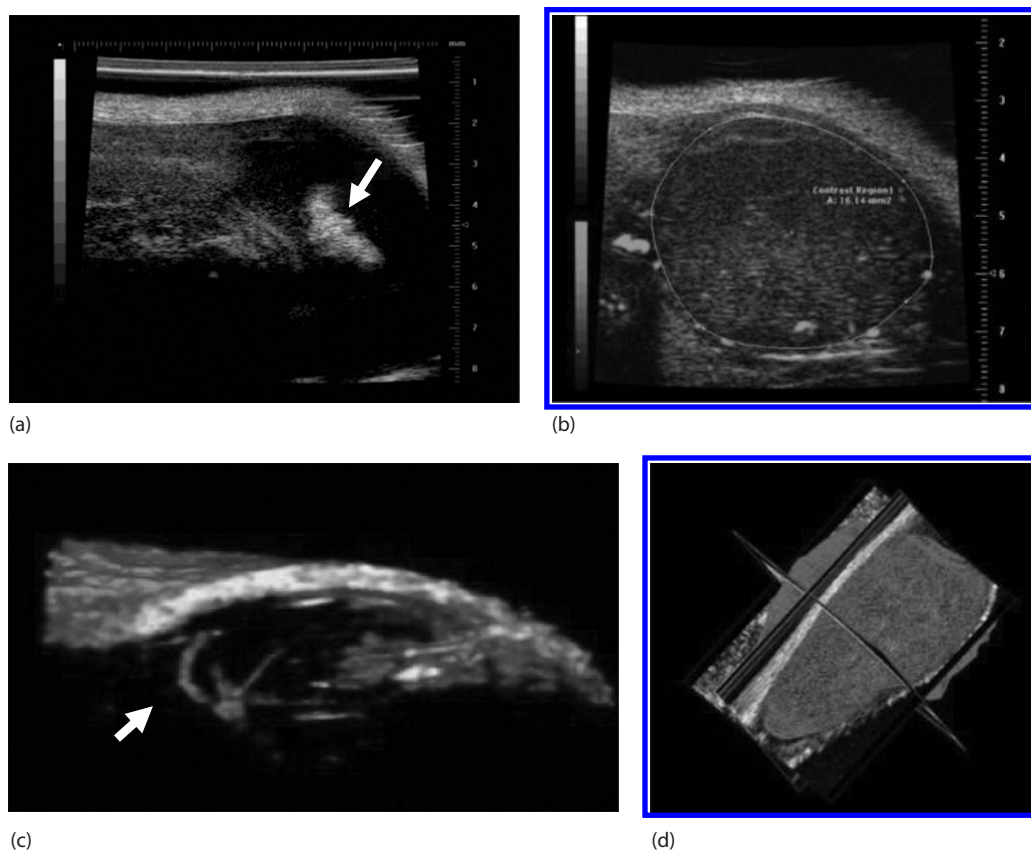
Figure 14.10   Micro Ultrasound images. (a) B-Mode image of pancreatic tumor in transgenic mouse model. B-mode is used to reveal the anatomical structure. A pancreatic tumor (arrow) is identified by micro ultrasound. (b) Image of a mice xenograft tumor injected non-targeted microbubbles. Microbubbles are the most commonly used contrast agent for ultrasound imaging. Non-targeted microbubbles can be used in evaluating microperfusion within a region of interest (ROI). Custom designed targeted microbubbles can be used to measure the expression of specific molecular markers. (c) 3D Power Doppler image of mouse lower hind limb. Power Doppler mode is used to measure the perfusion or the percentage of vascularity within a region of interest in ultrasound imaging. The branch-like structure where the arrow points is the primary vessel of the lower limb. (d) Image of a mouse xenograft tumor after 3D reconstruction. (Images provided by Van Andel Research Institute, Grand Rapids, MI.)

animals, usually rodents, and then sacrificing a certain number of the animals at times following dosing that represent each step in the ADME process. The animals are then either necropsied or sectioned, examined and radioactivity is quantitated with scintillation counters, or using Quantitative Whole Body Autoradiography (QWBA), respectively, to demonstrate where the drug distributed in high resolution and how it was excreted (see Figure 14.11) in a timely fashion. This traditional approach to ADME is a labor-intensive process that requires several groups of animals, with 4–6 animals per group and 5–10 groups, to avoid random variations in the data.

Positron emission tomography (PET), single photon emission computed tomography (SPECT), quantitative whole body autoradiography (QWBA), fluoroscopy, high-resolution ultrasound, optical and other imaging techniques offer numerous benefits in ADME studies. The co-registration of PET/CT and SPECT/CT imaging enables the measurement of rapid kinetic processes in real time and can therefore generate a more accurate picture of the ADME profile. Many of these imaging
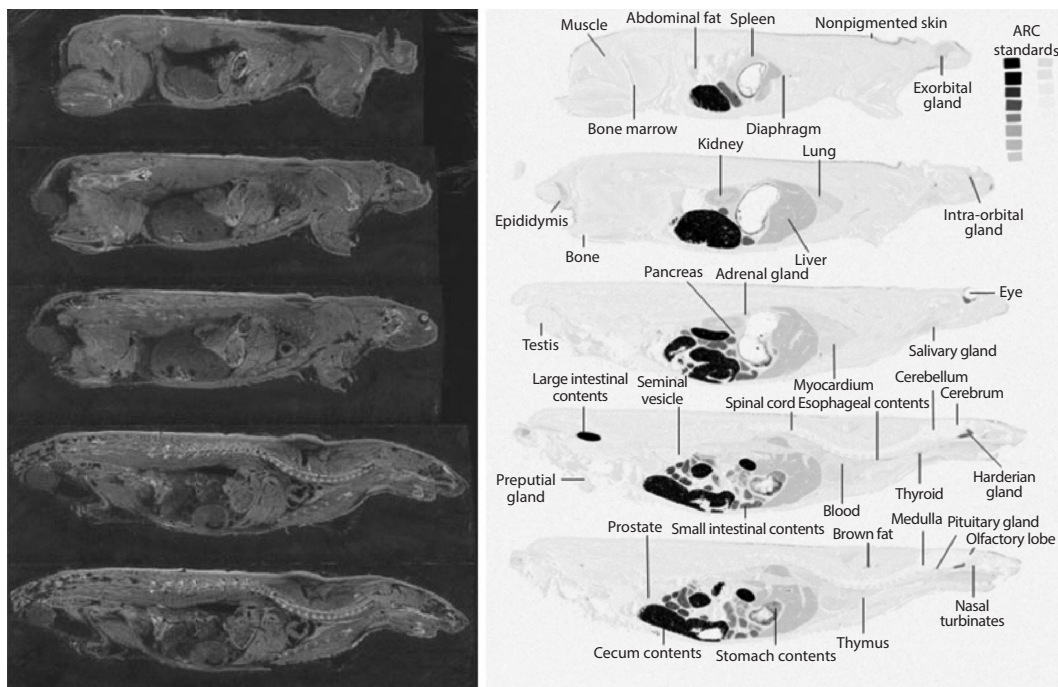
Figure 14.11 Quantitative whole-body autoradiography (QWBA) images of tissue distribution of ^{14}C radiolabeled test article in rat. Five levels of section specimen are collected per animal to obtain necessary tissues, organs, and biological fluids. The freeze dry sections are exposed to phosphor imaging screen for 4 days to capture scintillation light signals, which are converted into digital images to imaging data analysis of drug distribution in tissue and organs. (Image reconstruction provided by MPI Research Inc., Mattawan, MI.)

technologies allow the use of mice as animal models, thereby reducing costs. The continued survival, and analysis of the same subject animal model through entire imaging process also minimizes variability and ensures accuracy of data.

By attaching relatively short-lived medical isotopes such as SPECT and PET radiotracers to potential drugs, researchers can follow a prospective drug candidate through live animals to define ADME characteristics in real time. This means that one needs fewer animals to see how drugs work. In addition, in vivo imaging provides bio-distribution information in real-time, as compared to the extensive post-mortem evaluation procedures required for more traditional approaches. No longer does one have to use many animals for statistics, so it is quicker, cheaper and it also saves animals as the animals need not be serially sacrificed as in the traditional method.

In preclinical imaging studies, animals can be imaged with the same radioisotopes as used in the clinic for humans, but at dose levels adapted to the smaller subjects. As a result, successful pre-clinical studies in animals quickly translate to clinical studies on humans. Provided that small-animal imagers are indeed able to image small subjects with the same utility as humans in the clinic, the ability to use the same tracer in human clinical trial as those used in pre-clinical animal testing, can result in significant efficiency improvements and time and cost savings when translated into humans in clinical practice.

As a result of these new imaging modalities being available for preclinical development, PET and SPECT imaging have important roles to play in both the pre-clinical stages, as well as the human clinical research leading up to submission of a prospective drug to the FDA for marketing approval. In vivo molecular imaging permits earlier determination of whether a given group of drug

candidates will work or not in laboratory animal models. By using imaging to look at responses earlier in animal, safety can be further ensured and resources can be saved by abandoning drugs that do not translate to the human model in favor of those that do by early "go/no go" decisions.

In an imaging study of drug screen,[9] Zhang and his collaborators use microPET to evaluate the tumor-targeting ability of biologic molecules including three types of single-domain antibodies (sdAbs): sdAb itself (EG2, ~16 kDa, one antigen binding site,), pentabody (V2C-EG2, ~126 kDa, five antigen binding sites), and chimeric HCAb (cHCAb named EG2-hFc, ~80 kDa, two antigen binding sites). EG2, V2C-EG2, and EG2-hFc were all labeled with ^{64}Cu and used for imaging a human pancreatic carcinoma model, MIA PaCa-2, established in nude mouse for each construct. MicroPET/CT fused images suggested that the majority of EG2 and V2C-EG2 localized in the kidneys 1 hour after injection (Figure 14.12a and b). Both proteins were barely detectable in the tumor at 1 and 4 hours. In contrast, microPET/CT images of the mouse administered with EG2-hFc revealed gradual accumulation in tumor and gradual reduction in other organs for the observed period (up to 44 hours). No obvious kidney uptake was noticed (Figure 14.12c). In addition, good tumor/muscle contrast was observed after 20 hours, and the contour of the tumor in the PET image matches the true tumor shape. Whereas EG2 and V2C-EG2 localized mainly in the kidneys after i.v. injection, EG2-hFc exhibited substantial tumor accumulation. The outstanding tumor imaging of 64Cu-EG2-hFc is largely attributed to its long serum half-life, which is comparable to that of IgGs. The moderate size (~80 kDa) and intact human Fc make HCAbs a unique antibody format which may outperform whole IgGs as an excellent imaging and/or a potential therapeutic reagent.

On the other hand, microscopic imaging method is also increasingly being recognized as a valuable tool in ADME testing, which can be employed in both in vitro and in vivo preclinical studies. Microscope images can reveal multiple pieces of information on the cellular response to drug compounds in one experiment. This might include the simultaneous acquisition of data on drug-receptor binding and any morphological effects of drug treatment. The ability to use several protein-specific fluorescent probes in one experiment is a key enabling technique in high content imaging. Fluorescence microscopic imaging allows molecules beyond the resolution limit of the light microscope to be visualized.[20] Fluorescence microscopy is a key technique in clinical diagnostic as well as research settings. Confocal fluorescence microscopy, in particular, has become an essential tool central to the study of structural and molecular dynamics in living cells. Time-lapse imaging can be used to monitor downstream drug effects and, ultimately, excretion from the cell. Once images have been digitally captured, it is also possible to examine the data retrospectively in response to new questions about the drug compound. Microscopy images have the advantage that they are easily machine readable with appropriate image analysis software. This makes microscopy amenable to medium- to high-throughput analysis. Motorized and computer controlled microscopes are essential for automated image capture and for incorporation into medium to high throughput environments.

Bioluminescent probes have also been used for ADME evaluation. There have been several reports in the literature published, describing the synthesis and utility of bioluminescent probes that are substrates of CYP family of enzymes. CYPs are a critical focus of drug development efforts because they are the dominant drug-metabolizing enzymes. CYPs predominantly facilitate drug clearance and in certain cases activation of prodrugs into active compounds. The identification of CYP oxidation sites on newly designed drug molecules might guide redesign efforts so as to minimize breakdown by the CYP family enzymes while retaining its activity. Klaubert and colleagues presented a series of CYP specific luminescent probes that could help uncover specific CYP functions against a background of multiple CYPs in samples such as liver microsomes and cultured hepatocytes.[21]

It is ideal to have an imaging agent that selectively targets the specific organ or pathophysiologic lesions for best imaging contrast and diagnostic accuracy *in vivo*. Nevertheless, most of the dyes themselves are not target-specific. The in vivo performance of an imaging probe can be complicated
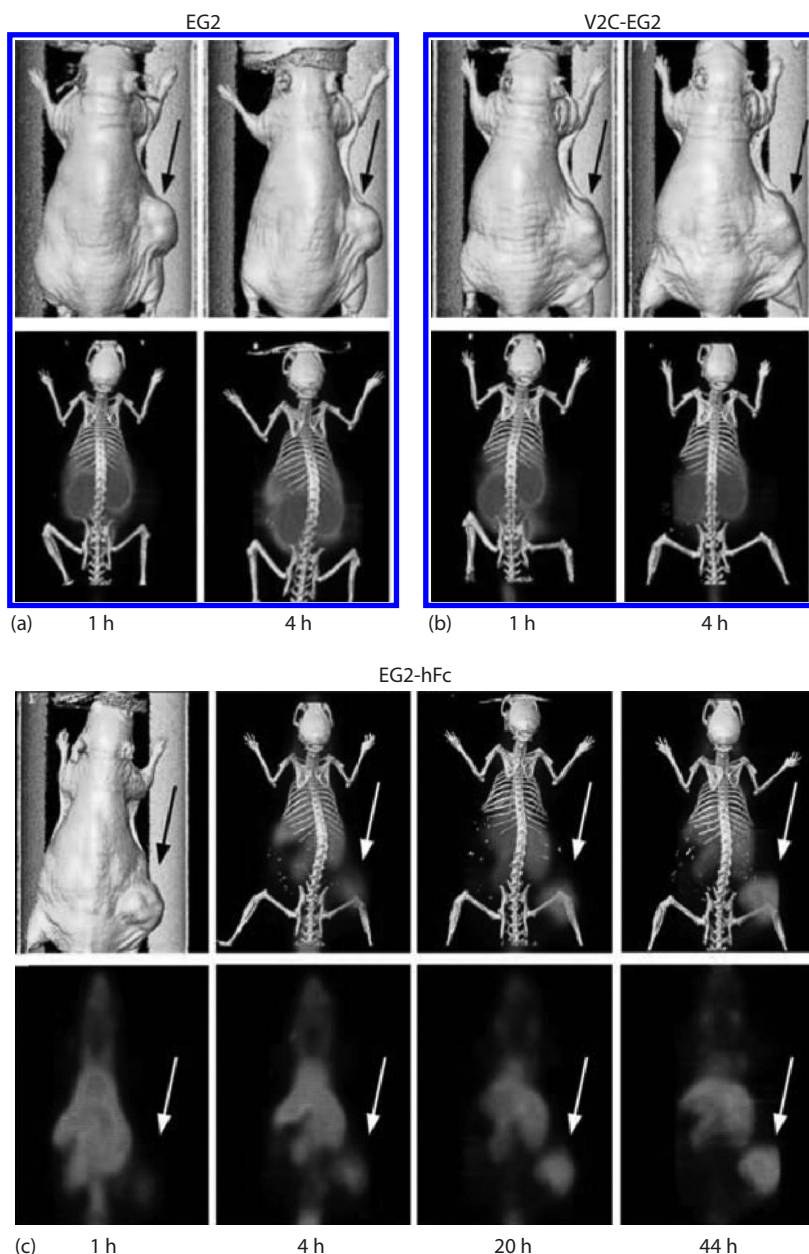
Figure 14.12 Fused microPET/CT images of human pancreatic carcinoma model MIA PaCa-2. Mice bearing established tumors were i.v. injected with ^{64}Cu-DOTAEG2 (a), 64Cu-DOTA-V2C-EG2 (b), and 64Cu-DOTA-EG2-hFc (c). For EG2 and V2C-EG2, the mice were imaged at 1, 4 and 20 h post-injection (20 h data not shown). For EG2-hFc, the mouse was imaged at 1, 4, 20, and 44 h post-injection. The top row in each sub-figure contains either surface rendering images performed using AmiraTM (Mercury Computer System Inc.) to show relative tumor location (arrows) (a–c at 1 h) or fused microPET/CT images (only c at 4, 20, and 44 h). The bottom row in each sub-figure contains either fused microPET/CT images (a and b) or microPET images (c). (Images adapted and modified from Bell, A. et al., *Cancer Lett*., 289, 81, 2009.)

by its interactions with many bio-molecules, membranes and related cellular permeability or tissue penetration as well as pharmacokinetic processes including absorption, distribution, metabolism, and excretion (ADME). Therefore, it has been challenging to discover and develop an optimal imaging agent for *in vivo,* namely targeted imaging.

Preclinical Imaging in Oncology

Cancer drug discovery is a relatively long process. Many imaging techniques have been routinely used in the drug discovery process to directly monitor the drug in blood and tumor tissues to evaluate the effects of the drug treatment in the context of tumor. Molecular imaging recently emerges with increasing popularity as it can be used to monitor the changes at the molecular level in vivo, and it can help in evaluating treatment efficacy much earlier than traditional clinical endpoint. For example, [18]FDG-PET imaging is one of the most powerful molecular imaging techniques so far available for clinical use to detect, staging, monitoring, and evaluating the prognosis of cancer.[22] In clinical practice, [18]FDG could offer adequate contrast and tumor detection in several cancers, such as, lung, colon, breast cancer, and lymphoma. However, the clinical result is not satisfying in other cancers, such as, renal, head and neck, prostate and pancreatic cancer.[23] Therefore, new imaging probes is need for early tumor detection in both preclinical and clinical research. Recently, one of tumor imaging study aims at verifying the capability of [64]Cu (a positron emitter, half-life, 12.7 hours) radiolabeled bevacizumab, an anti-cancer therapeutic antibody targeted tumor angiogenesis, in detecting different types of tumors[24] in early stage of tumor model and comparing with the gold standard [18]FDG. The project used different strategy from [18]FDG in order to accurately detect tumors in earlier stage and effectively decrease the non-tumor related hot spots in the background of the [18]FDG-PET imaging (also see Figure 14.4). This preliminary imaging result illustrates that ^{64}Cu-bevacizumab would have superior properties as a new generation of tumor detection probe compare to [18]FDG probe in preclinical imaging study.

The imaging techniques can also be used to evaluate efficacy and therapeutic response of anti-cancer drug candidate in the same tumor model so as to minimize the subject-to-subject variability and reduce the number of animals required by more traditional methods. One efficacy imaging study represents a powerful way for tracking adoptively transferred T cells and studying their in vivo distribution and therapeutic effect in DUC18/CMS5 tumor model by microPET imaging.[11] In the efficacy study, the anti-Thy1.2 antibodies conjugated to 1,4,7,10-tetraazacyclododecane-tetraacetic acid (DOTA) and radiolabeled with ^{64}Cu were administered to three groups of BALB-Thy1.1 mice on days 4, 7, or 14 post-DUC18 T cell transfer. The imaging probe successfully detected the transferred cells in tumor tissue and imaged the size and shape of the tumors in the same living subject for therapeutic evaluation. Information obtained from the in vivo T cell trafficking studies could aid in designing protocols that would enhance the efficacy of tumor-specific T cells (see Figure 14.13). The cutting-edge imaging technology helps us further understanding in the area of trafficking behavior of adoptively transferred tumor-specific T cells and improve efficacy study with much less animal numbers since the same animal can be repeatedly imaged in different days post treatment when tumor growing or be ablated during the treatment.

On the other hand, various optical imaging techniques can be used in combination, either simultaneously or sequentially, to provide information from cancer cells and tumor tissues in living animal models. Among them, both fluorescence and bioluminescence imaging techniques have found wide applications for in vivo tumor optical imaging in mouse models (also see Figure 14.9). These approaches afford the collection of convenient, frequent visualization and measurement of tumor biomarkers in a real time, sensitive, and non-invasive way.

One of most successful imaging modes for preclinical imaging study is optical imaging technique which has been largely exploited to monitor the progress of practically all strategies for gene and cell therapy, regardless of vector or indication.[25–27] The ability to image host responses to both

4 days post treatment   8 days post treatment   15 days post treatment
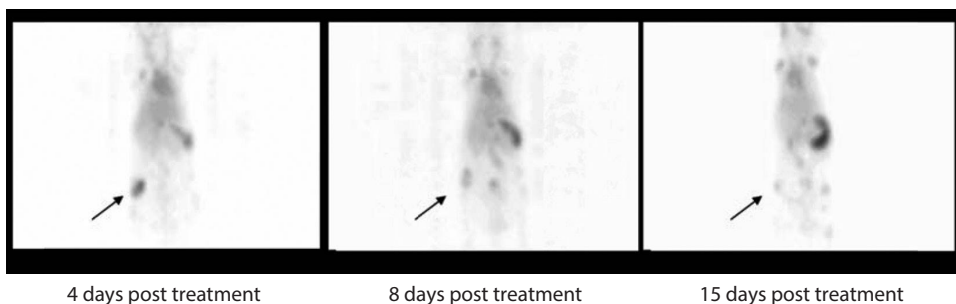
Figure 14.13 microPET images of cell therapy and tumor ablation. ^{64}Cu-labeled-Thy1.2 antibody specifically detects the tumors (arrow), major lymph nodes and spleens in DUC18/CMS5 tumor mouse model. Two-dimensional images are shown to represent an experimental mouse in the 4 days, 8 days, and 15 days post treatment with adoptively transferred tumor-specific T cells. Black arrows were used to indicate tumors for the purpose of presentation. (Images adapted and modified from Matsui, K. et al., *Nucl. Med. Biol.*, 31, 1021, 2004.)

vector administration and therapeutic interventions, provides crucial data for clinical translation. By using a combination of bioluminescence and fluorescence, the efficacy of gene transfer as well as therapeutic response can be monitored simultaneously. A variety of vector studies which include viral vectors, plasmids have been combined with bioluminescence for analyzing and understanding the effect of gene transfer to a variety of tissues and variety of physiological conditions such as tumors, autoimmune disorders, neurological conditions, etc.[28–36]

One of the earliest uses of optical imaging probes for molecular imaging purposes involved the use of bioluminescent reporter probes.[37,38] The fusion-gene reporter system is an indirect imaging strategy where two or more genes are fused together under the control of a single common promoter giving rise to a single polypeptide or transcript. The output of fusion reporter genes is often in form of fluorescence from a light emitting gene such as GFP or RFP as well as other reporters such as the luciferase reporter which generates fluorescence upon addition of a substrate such as luciferin.[39,40] This system provides the ability to noninvasively obtain molecular information in systems ranging from a single live cell to the multicellular environment of an animal holds tremendous potential for studying cancer metastasis, efficacy of drug therapy and many other applications in a preclinical imaging in laboratory animal (see Figure 14.14). They can also be used for imaging of other disease models such as inflammation as demonstrated by Yaghoubi et al. using a mouse model of arthritic induced inflammation; the authors were able to successfully image migration of T-cells into the inflamed paws.[41] Another use of reporter genes is the ability to study drug protein interactions. A drug can activate a specific protein whose expression can be analyzed by optical reporters. Enzymes are a class of proteins, whose expression levels in various diseases models can be easily monitored by use of fusion reporters. Ray et al. developed a multimodality sensor that comprised a bioluminescent, a fluorescent and a PET reporter gene linked together by a four amino-acid spacer (DEVD), a classical caspase 3 cleavage site. The activation of the caspase 3 through regulators of apoptosis could be easily visualized by all the three modalities including bioluminescence and fluorescence. This system could be used for screening of apoptosis activators/inhibitors in a preclinical drug development.[42] Activatable probes as described above are an interesting class of molecular imaging probes.[43] These probes are designed to generate a signal in response to specific biological stimuli. The signal generated is amplified and observed by use of optical imaging agent, which is one of the most popular modalities utilized. Most activatable probes are based on fluorescence activation methods. The idea is that in close proximity a donor (fluorophore) and a quencher (acceptor) silence each other optically because of a variety of quenching phenomenon such as resonance energy transfer (FRET), dark-quenching mechanisms, and nanoparticle-based
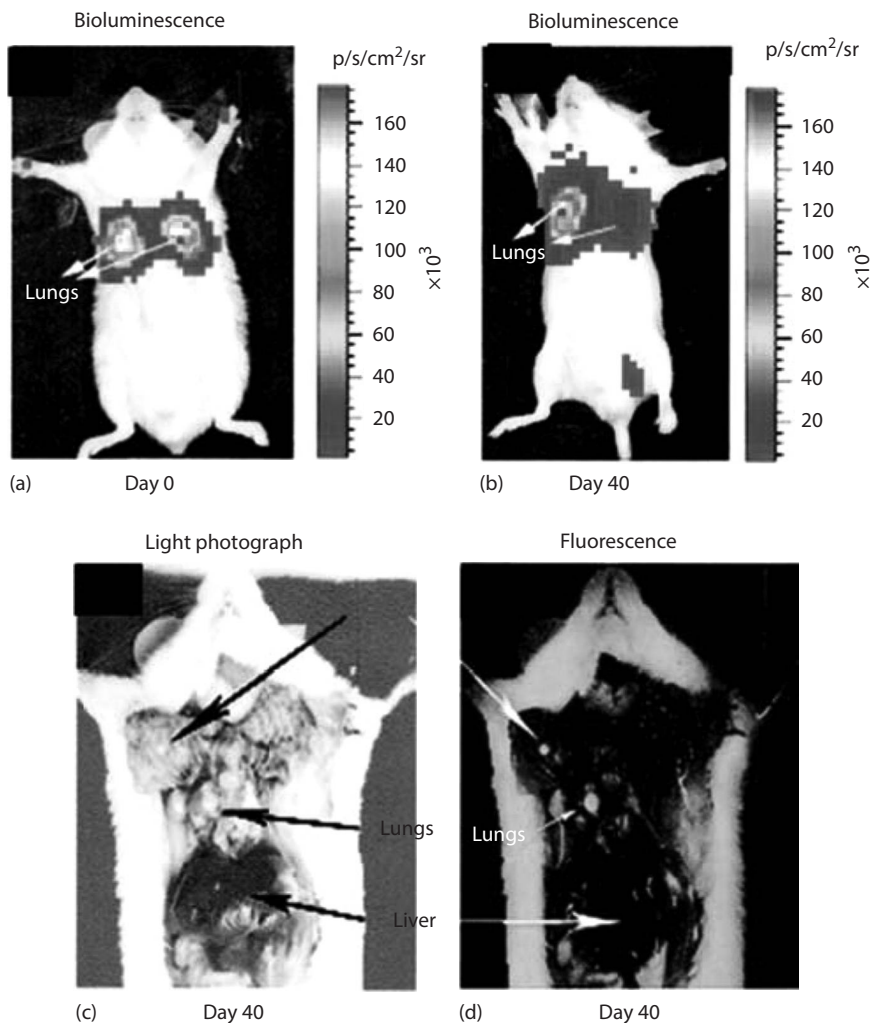
Figure 14.14 Multimodality imaging of metastasis of A375M cells stably expressing the hrl-mrfp-ttk fusion reporter gene in living mice. (a) Bioluminescence imaging of an SCID mouse injected with A375M cells expressing the hrl-mrfp-ttk vector at day 0. A375M cells stably expressing the triple fusion were injected via tail-vein in an SCID mouse and 2 h later imaged for bioluminescence signal. Prominent bioluminescence signal was found from the region of both the lungs. (b) Bioluminescence imaging of the same SCID mouse at day 40. At day 40, the same mouse was imaged and relatively high bioluminescence was found from the left lung region and moderate signal from the right lung region. A faint bioluminescence signal was also present from the right pelvic region. (c) Light photograph of the same SCID mouse after sacrifice and organ exposure (d) whole body fluorescence imaging of the same SCID mouse. Fluorescing metastatic tumors were found in lung and chest regions that correspond with the bioluminescence and PET images. (Modified from Ray, P. et al., *Cancer Res.,* 64, 1323, 2004. With permission.)

surface energy transfer.[44–46] These probes often described as "molecular beacons" are activated in the presence of specific biomolecules or chemical stimuli, which generate an amplified fluorescence signal. The distance between the donor and acceptor molecules thus plays an important role when designing useful imaging probes.

The most popular activatable probes used for cancer imaging are protease-activatable imaging probes.[47] Proteases are enzymes which can break down proteins by hydrolyzing the peptide

bonds that link the amino acids in the polypeptide chains making up the protein. Proteases are known to be overexpressed under different pathologic conditions including cancer. To date, various activatable probes have been developed to detect and image representative cancer-related proteases, such as matrix metalloproteinases (MMPs), cathepsins, and caspases. MMPs are a family of zinc-dependent endopeptidases that play key roles in tumor invasiveness, metastasis, and angiogenesis.[48,49] A dual-labeled MMP-7 activatable peptide probe was designed for potential use in imaging MMP-7 activity in tumors. It comprised of a quencher NIRQ820 linked to a donor Cy5.5 through a linker (GVPLSLTMGC polypeptide chain) which is a well-known substrate for MMP 7. In vitro studies pointed out the specificity of the peptide to be cleaved by MMP 7 leading to increase in optical signal following incubation.[50] Another class of quenchers used for design of activatable probes involves use of dark quenchers which have no known fluorescence from the visible to the NIR spectrum. Black Hole Quencher (BHQ) dyes are one of the commercially available dark quenchers and are able to permit efficient quenching. Studies have demonstrated the synthesis of an MMP-13 activatable peptide probe was designed using a combination of the known MMP-13 substrate GPLGMRGLGK and Cy5.5 and BHQ-3 (see Figure 14.15). The study[51] showed a 32 times increase in the optical signal following incubation with MMP 13. The specificity of the peptide substrate was also observed following decease in optical signal when co-treated with a MMP 13 inhibitor. Tsien and colleagues developed a similar probe called as activatable cell penetrating peptide. This system allows for the intracellular transportation of the probe following hydrolysis by extracellular peptidases. The transportation of the probe only occur following protease specific activation. In vivo studies with ACCP in mice with HT90 tumors as well other models such as a transgenic breast cancer model, showed threefold increases in tumor uptake following injection.[52–54]

Apoptosis or programmed cell death allows plays an important role following cancer treatment. Noninvasive approaches to monitor apoptosis will allow for determining the efficacy of existing as well new antitumor therapies in a preclinical and clinical setting. Caspase families of enzymes are known mediators of apoptosis.[55,56]

Activation of imaging probes linked with caspase target polypeptides could allow for imaging of apoptosis in cells (see Figure 14.16). Piwnica-Worms et al. developed a cell-permeable, caspase-activatable probe consisting of a cell-penetrating peptide, Tat peptide (Ac-rkkrrorrr) or KKKRKV, conjugated to a caspase-3/7 substrate, DEVD). In vitro enzyme assays showed that the probe was preferentially activated by the effector caspases 3 and 7. In addition, the probe was able to detect apoptosis in animal models, including parasite-induced apoptosis in human colon xenografts and N-methyl-D-aspartate-induced apoptosis in an in vivo model of glaucoma.[57] Macromolecules such as polymer based systems, dendrimer, and antibodies have been used for development of molecular imaging agents for cancer and other disease models. In Figure 14.17, Weissleder and colleagues developed a probe consisting of multiple Cy5.5 molecules attached to a methoxy-PEG-grafted poly-L-lysine copolymer (PGC). The lysine on the polymer acts as a target for various peptidases such as cathepsins and caspases.[58] Enzyme incubation leads to a 12-fold increase in signal under in vitro conditions. Dendrimer and other macromolecules such as chitosan based polymers have been used in a similar manner for molecular imaging of cathepsin as well as caspases under in vitro as well as in vivo conditions. Antibodies have also be studies for developing imaging moieties to specific targets in cancer. Antibody imaging can provide a sensitive, noninvasive means for characterizing cell surfaces. Kobayashi and colleagues have developed a series of antibody conjugated optical imaging agents. Indocyanine green (ICG) was conjugated to a variety of monoclonal antibodies targeted towards a variety of receptors such as anti-CD25, anti-EGFR1 (epidermal growth factor receptor 1), and anti-HER2 (human epidermal growth factor receptor 2). These conjugates are nonfluorescent as the fluorescence is quenched because of the proximity of the antibody and the fluorescent dye. But intracellular uptake of the antibody by tumor cells and further breakdown allows for fluorescence recovery of the dye for imaging tumor cells. This methodology has been used successfully to image
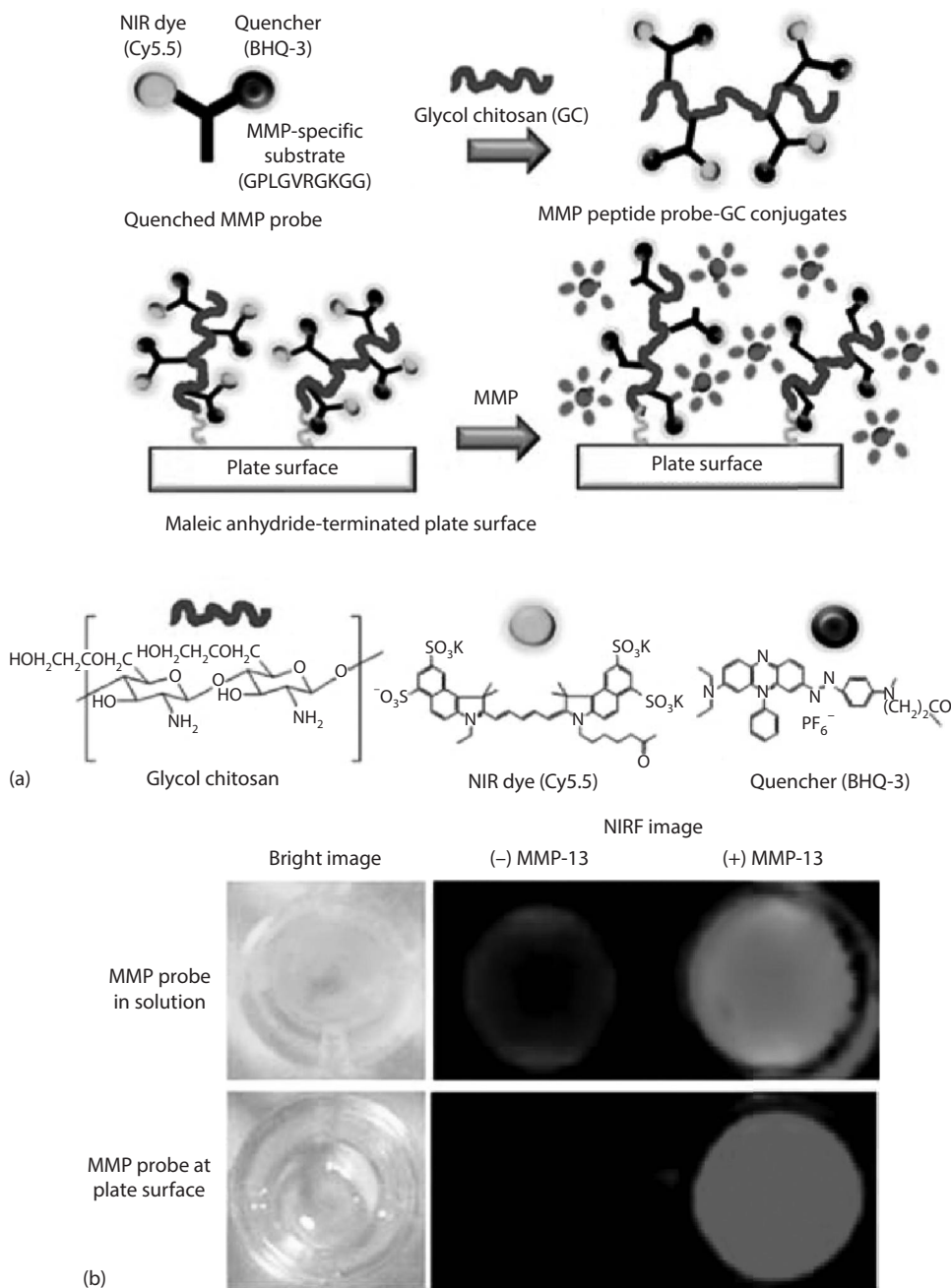
Figure 14.15   Synthesis of an MMP-13 activatable peptide probe. (a) Schematic diagram of MMP diagnostic kit (MMP-D-KIT) for "one step" detection of MMP activity. Immobilization of MMP peptide probe–GC conjugates onto 96-well plates. (b) Bright and NIR fluorescent images of 96-well plates containing MMP peptide probe–GC conjugates in the immobilized phase (MMP-D-KIT) and those in the solution, respectively. (Reprinted with permission from *Bioconjug. Chem.,* 21(10), 1378. Copyright 2010 American Chemical Society.)

(a)

(b)

(c)   Time: 50 min after probe
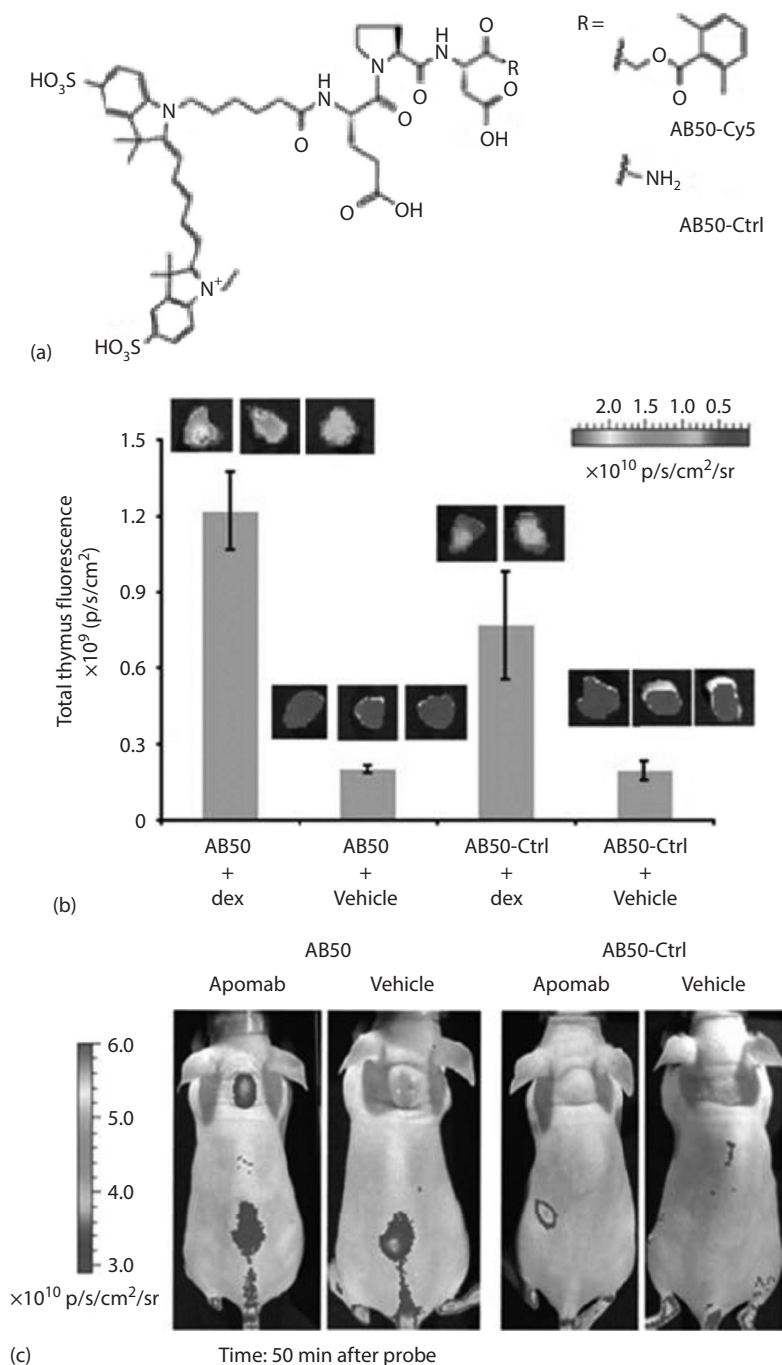
Figure 14.16 Activation of imaging probes linked with caspase target polypeptides could allow for imaging of apoptosis in cells. (a) Structures of the optimal caspase probe AB50-Cy5 and its control counterpart AB50-Ctrl that contains an amide in place of the AOMK reactive group. (b) Quantification of total Cy5 fluorescence in thymi from mice treated with AB50-Cy5 or AB50-Ctrl for 50 min. (c) Noninvasive images of tumor-bearing mice treated for 12 h with Apomab or vehicle control. (Modified with permission from Macmillan Publishers Ltd. *Nat. Med.,* Edgington, L. E. et al., Noninvasive optical imaging of apoptosis by caspase-targeted activity-based probes, 15, 967–973, Copyright 2009, on behalf of Cancer Research UK.)

tumors over expressing certain markers under in vivo conditions.[59–63] Integrins are class of proteins which have a wide variety of functions in mediating cell–cell communication and cell–extracellular matrix interactions. They have a wide variety of roles including promoting angiogenesis, cell migration and cell survival.[64,65] They have been implicated in a wide variety of cancers such as glioblastomas, melanomas, breast cancer, head and neck tumors amongst others. The mot extensively studied integrin is the αvβ3 integrin. It is known to bind to a specific ligand a three amino acid polypeptide chain consisting of arginine-glycine-aspartic acid (RGD). This polypeptide is associated with many receptors such as vitronectin, fibronectin and thrombospondin that have been associated with many cancers such as brain, ovarian, breast, prostate, etc.[66–69] Imaging of αvβ3 integrin can
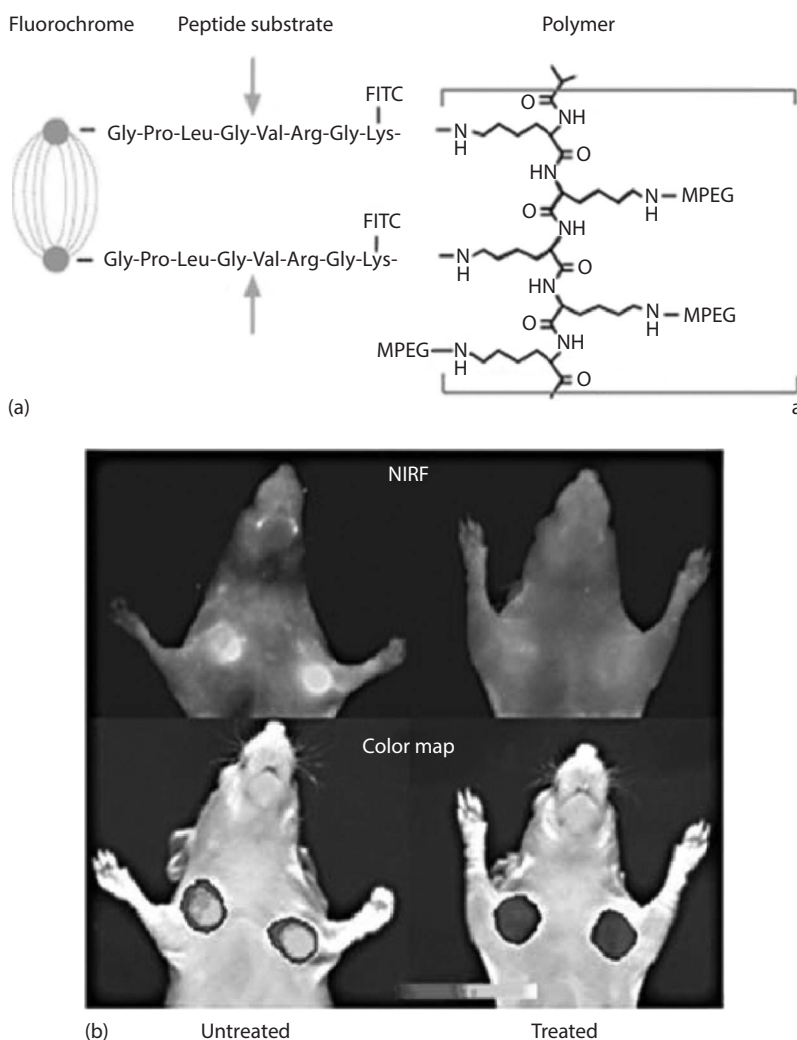
Figure 14.17 Polymer based optical probe for optical imaging in mice. (a) The MMP-2-sensitive imaging probes consist of three structural elements: The quenched NIR fluorochrome, an MMP-2 peptide substrate and a graft copolymer (methoxy-polyethylene-glycol-derivatized poly-L-lysine). In vivo NIRF imaging of HT1080 tumor-bearing animals. (b) The top row shows raw image acquisition obtained at 700 nm emission. Untreated (left), treated with 150 mg/kg prinomastat, twice a day, i.p. for 2 days (right). The bottom row shows color-coded tumoral maps of MMP-2 activity superimposed onto white-light images (no treatment [left], prinomastat treatment [right]).   (*Continued*)
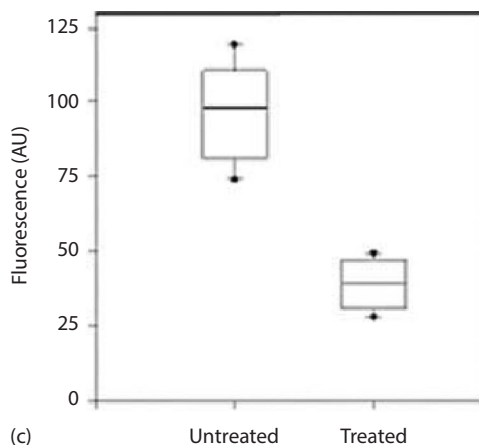
Figure 14.17 (*Continued*) Polymer based optical probe for optical imaging in mice. (c) Quantitative image analysis of all 20 tumors. Tumoral NIRF signals are shown in a box plot (bars indicate 10th and 90th percentile). The difference in imaging signal between the two groups was statistically significant ($P < 0.0001$). (Reprinted with permission from Tung, C. H. et al., Preparation of a cathepsin D sensitive near-infrared fluorescence probe for imaging, *Bioconjug. Chem.,* 10, 892. Copyright 1999 American Chemical Society.)

provide a lot of information about the tumor at the structural, functional and even molecular level. Chen et al. developed an NIRF probe conjugated to RGD for imaging of brain tumors.[70] In vivo studies demonstrated high tumor contrast of the targeted probe and good delineation of the tumor. Subsequent studies have shown the use of a wide variety of contrast agents conjugated with the cRGD peptide for imaging a variety of tumor models.[71,72] Molecular modeling studies have identified preferential interaction of the cRGD sequence with the β3 subunit of integrins relative to the αv subunit, suggesting that most probes mainly target the β3 integrin but not the heterodimer αvβ3. RGD peptides have also been used for imaging of ischemia in mouse models. In the cardiovascular setting, angiogenesis is triggered by hypoxia and ischemia, and its major consequence in tissues is the restoration of perfusion and oxygenation. RGD dendrimers have been developed for multi-modal imaging of limb ischemia in mice.[73–78] The newer research focuses on developing multimeric compounds for imaging tumors including multivalent ligand-receptor interactions for improved molecular imaging. Similar approach has been used for imaging other overexpressed biomarkers such as folate receptors for image guided resection of ovarian cancer. Van Dam and colleagues reported the development of folate receptor targeted fluorescent probe, see Figure 14.18. The folate molecule was conjugated to FITC for image of folate receptor positive tumors (FR +ve). The newly designed molecular imaging probe was used under intra operative conditions for identification of folate positive tumor cells for possible resection. In the pilot study, the injected probe was found to be safe and offer specific and sensitive real-time identification of tumor tissue during surgery in patients with ovarian cancer and the presence of FR-α–positive tumors.[79]

The use of targeted fluorescent agents could provide a paradigm shift in surgical imaging as it allows an engineered approach to improving tumor staging and the technique of cytoreductive surgery and thereby improving the outcome in various cancers.

Nanoparticles have also been utilized for developing agents for fluorescence imaging of cancer and other modalities. Metal based nanoparticles such as quantum dots and gold nanoparticles have been investigated extensively as agents for optical imaging. These possess unique characteristics such as high photostability, low quenching properties and high luminosity.[80,81] Quantum dots are a class of nanoparticles made from semiconductor metals which based on size and composition can
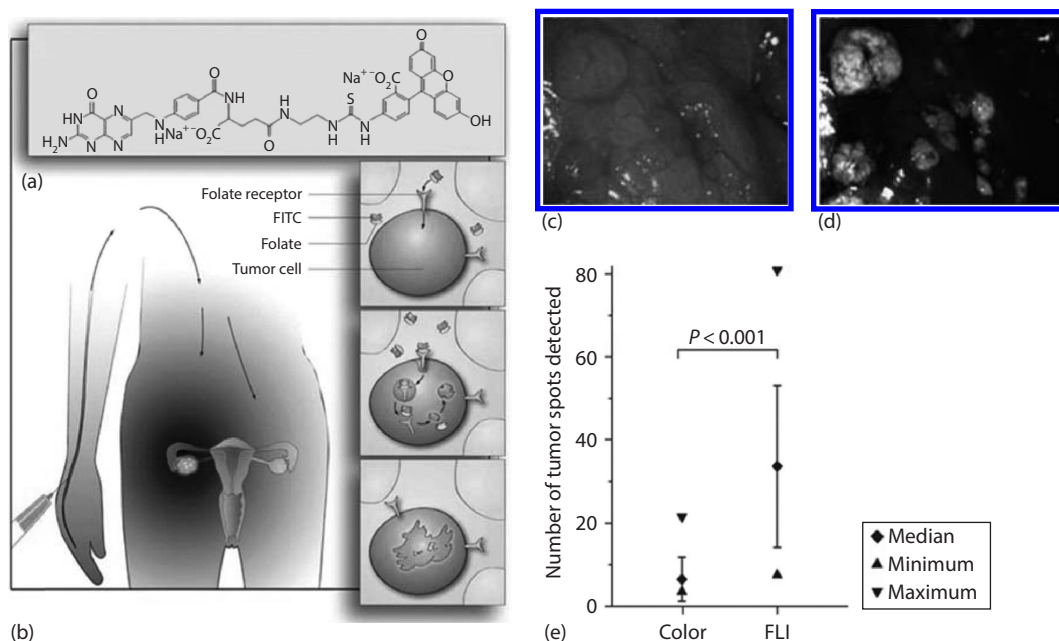
Figure 14.18   Folate receptor targeted fluorescent probe. (a) Folate is conjugated through an ethylenedi-amine spacer to fluorescein isothiocyanate (FITC), resulting in folate-FITC, with a molecular weight of 917 kDa. (b) A schematic presentation of the targeting of ovarian cancer. Folate-FITC is targeted toward FR-α and internalized upon binding, shuttling folate-FITC into the cyto-plasm. Color image (c) with the corresponding tumor-specific fluorescence image (d) of a rep-resentative area in the abdominal cavity. (e) Scoring was based on three different color images (median 7, range 4–22) and their corresponding fluorescence images (FLI) (median 34, range 8–81); $P < 0.001$ by five independent surgeons. (Reprinted with permission from Macmillan Publishers Ltd. *Nat. Med.*, van Dam, G. M. et al., Intraoperative tumor-specific fluorescence imaging in ovarian cancer by folate receptor-alpha targeting: First in-human results, 17, 1315–1319, Copyright 2011, on behalf of Cancer Research UK.)

generate light over wide spectrum ranging from visible to NIR.[82,83] They also have broad absorption windows, which makes QDs well-suited for multi-colored imaging applications.

Harrell et al.[84] used nontargeted QDs to demonstrate that increased lymph flow precedes tumor metastasis in a melanoma xenograft model. Medintz et al. developed a series of activatable QD-based probes to detect proteolytic activity of enzymes such as collagenase, capoase-1, thrombin and chymotrypsin.[85–87] These were developed by metal affinity based self-assembly of polypeptide sequences. An interesting concept would be to attach multiple fluorescent dyes on the surface of the QDs. The increasing number of the fluorescence molecules proportionally enhances the overlap integral between the QD and the fluorophore acceptor and can reduce their PL emission. The linker between the QD and fluorophore can be specifically designed to be the substrate of an enzyme of interest. Addition of the enzyme will lead to fluorescence recovery allowing for imaging of specific biomarkers. Antibodies conjugated to QDs have also been used for detection of tumors. Nie and col-leagues have synthesized QDs targeted towards prostate cancer cells by use of antibodies recognition the prostate membrane surface antigen (PSMA). The fluorescence imaging indicated the high tumor targeting after the injection of QD-PSMA Ab conjugates. But there was an issue with poor penetra-tion and high autofluorescence following injection of the conjugates.[88] Recent reports from Tada and colleagues have demonstrated the use of QD for imaging of HER2 positive tumors. The authors conjugated monoclonal antibodies targeting the HER2 receptor and show high tumor uptake using fluorescence imaging.[89] Other approaches for molecular imaging involve use of bioluminescence

based detection and fluorescent proteins conjugated to QDs. Bioluminescence resonance energy transfer (BRET) based imaging moiety was developed by Rao and colleagues. The authors' conjugated Renilla luciferase mutant (Luc8) fused with an MMP2 substrate polypeptide sequence to QDs. The addition of MMP-2 to this pre-assembled QD-substrate-Luc8 reduced the BRET ratio and enabled the detection of MMP-2 activity with high sensitivity.[90,91] QDs have also been used for mapping of sentinel lymph nodes, a site for metastatic tumorigenic cells following primary tumor development. Frangioni and Bawendi first reported the use of NIR QDs in labeling lymph nodes in living subjects. Experiments performed in small and large animals such as mouse and pigs allowed for high resolution imaging of sentinel lymph nodes using NIR CdTe/CdSe QDs. The mapping allowed for resection of the lymph node up to a 1 cm depth indicating the usefulness of SLN mapping for fluorescence based resection of tumorigenic lymph nodes.[92–95] Peptide conjugated to QDs have allowed for active targeting and subsequent imaging of tumors. Ruoslahti and colleagues have reported the discovery of novel peptides targeting the endothelium and lymphatic vessels surrounding the tumor. Peptides such as CGFECVRQCPERC (denoted as GFE) which binds to membrane dipeptidase on the endothelial cells,[96] KDEPQRRSARLSAKPAPPKPEPKP KKAPAKK (denoted as F3) preferentially binds to blood vessels and tumor cells in various tumors.[97] The peptide of CGNKRTRGC (denoted as LyP-1) which recognizes lymphatic vessels and tumor cells in certain tumors conjugated with QDs have shown to be extremely encouraging for fluorescence based imaging of various types tumors (see Figure 14.19).[98,99] Chen et al. have shown the use of cadmium as well as non-cadmium based integrin $\alpha v \beta 3$ targeted QDs for imaging of gliomas in mice models. QD were conjugated to RGD peptide for imaging $\alpha v \beta 3$ positive tumors (Figure 14.20). These QDs showed high concentration in tumor sites and immunofluorescence studies confirmed that the majority of the QD fluorescence signal in the tumor colocalized with the tumor vessels.[100] The high efficiency of conjugation for the synthesis of QDs and peptides along with the good sensitivity and specificity of these conjugates to image tumors warrants the further development of such agents for in vivo molecular diagnosis of tumor, including early detection as well as image guided resection of tumor via surgery.

(a)   (b)
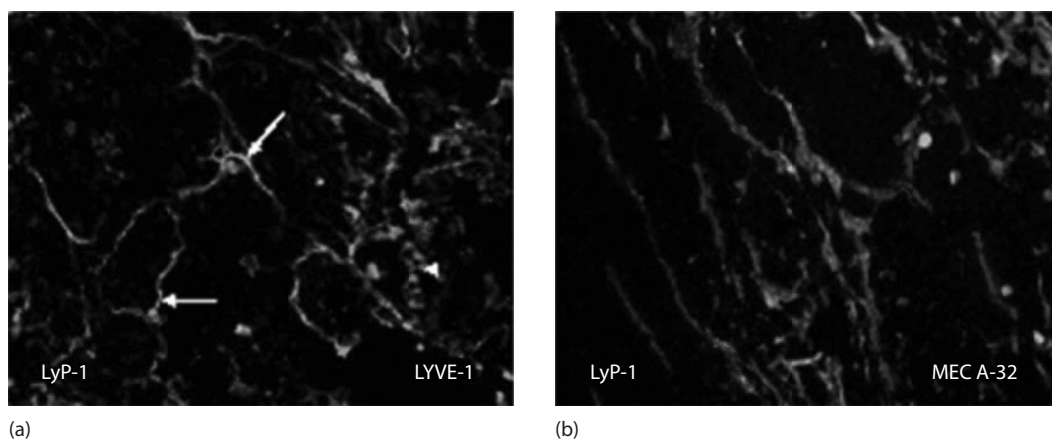
Figure 14.19 Tumor-homing peptide, LyP-1, recognizes tumor lymphatic vessels and some tumor cells, but not tumor blood vessels. Fluorescein-conjugated LyP-1 peptide was injected into the tail vein of MDA-MB-435 tumor-bearing mice. After 15 min of circulation, the tumor was excised and prepared for the immunohistological analysis. LyP-1 peptide (in a and b) homes to the lymphatic vessels and some tumor cells (*arrowhead* in a) within the tumor colocalizing with the lymphatic marker, LYVE-1, (*arrows*), but not with the blood vascular marker, MECA-32 (in b). (Reprinted from Laakkonen, P. et al., *Ann. NY Acad. Sci.,* 1131, 37, 2008. With permission.)
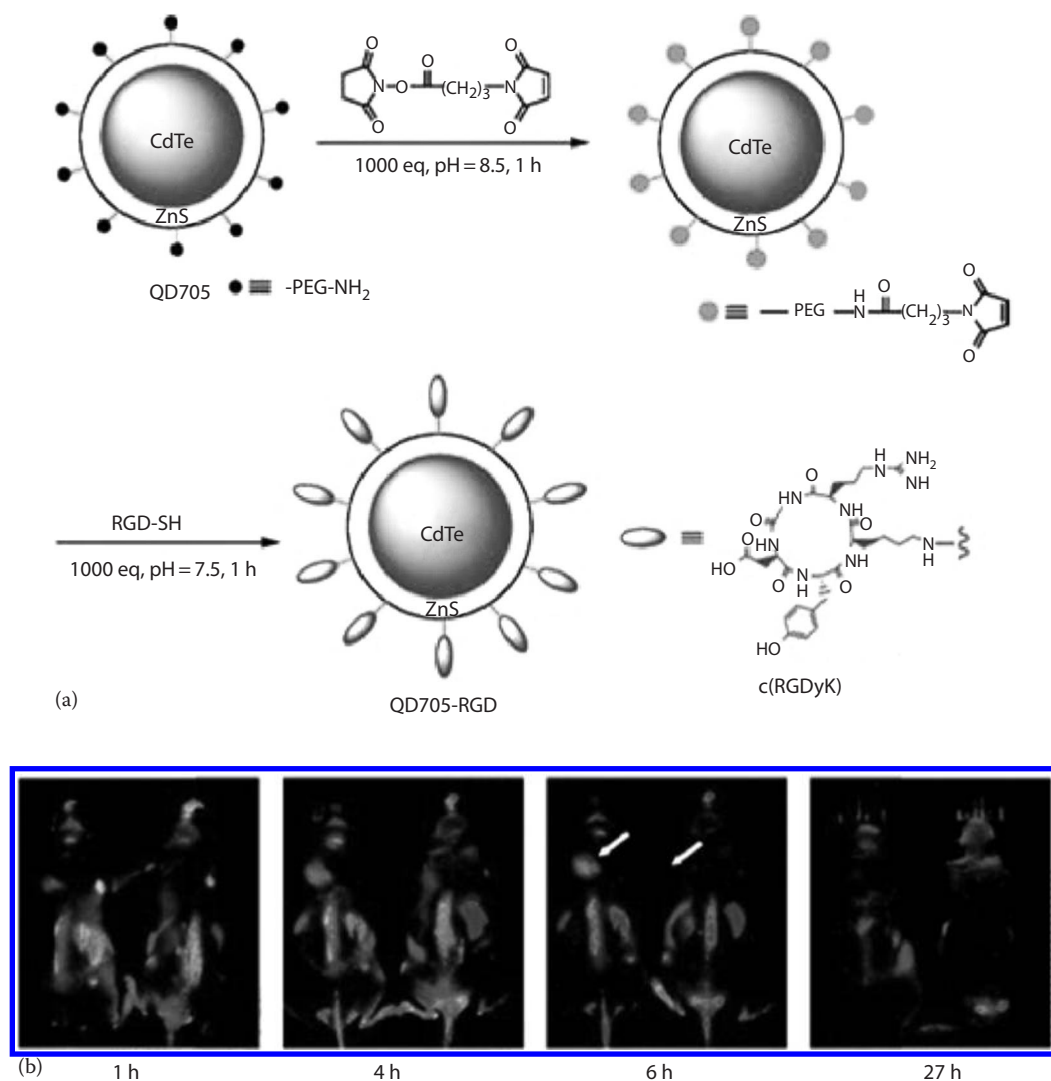
Figure 14.20 RGD peptide for imaging αvβ3 positive tumors. (a) Synthesis of QD705-RGD, PEG denotes poly(ethylene glycol) (MW 2000) (b) in vivo NIR fluorescence imaging of U87MG tumor-bearing mice (left shoulder, pointed by white arrows) injected with 200 pmol of QD705-RGD (left) and QD705 (right), respectively. All images were acquired under the same instrumental conditions. The mice autofluorescence is color coded green while the unmixed QD signal is color coded red (in original). Prominent uptake in the liver, bone marrow, and lymph nodes was also visible. (Modified with permission from Cai, W. et al., Peptide-labeled near-infrared quantum dots for imaging tumor vasculature in living subjects, *Nano Lett.*, 6(4), 669. Copyright 2006 American Chemical Society.)

Gold nanoparticles (AuNPs) have been shown to be promising candidates for biomedical applications. They are biocompatible, easily functionalized for attachment of a variety of ligands such as DNA, peptides, proteins, and antibodies for developing target specific agents. Their unique surface plasmon resonance absorbance allows for distinctive optical properties which can be used for sensing and imaging biomolecules. It has been noted in the literature that attachment of fluorescent molecules to the gold nanoparticle surface allows for unique interactions leading to the development
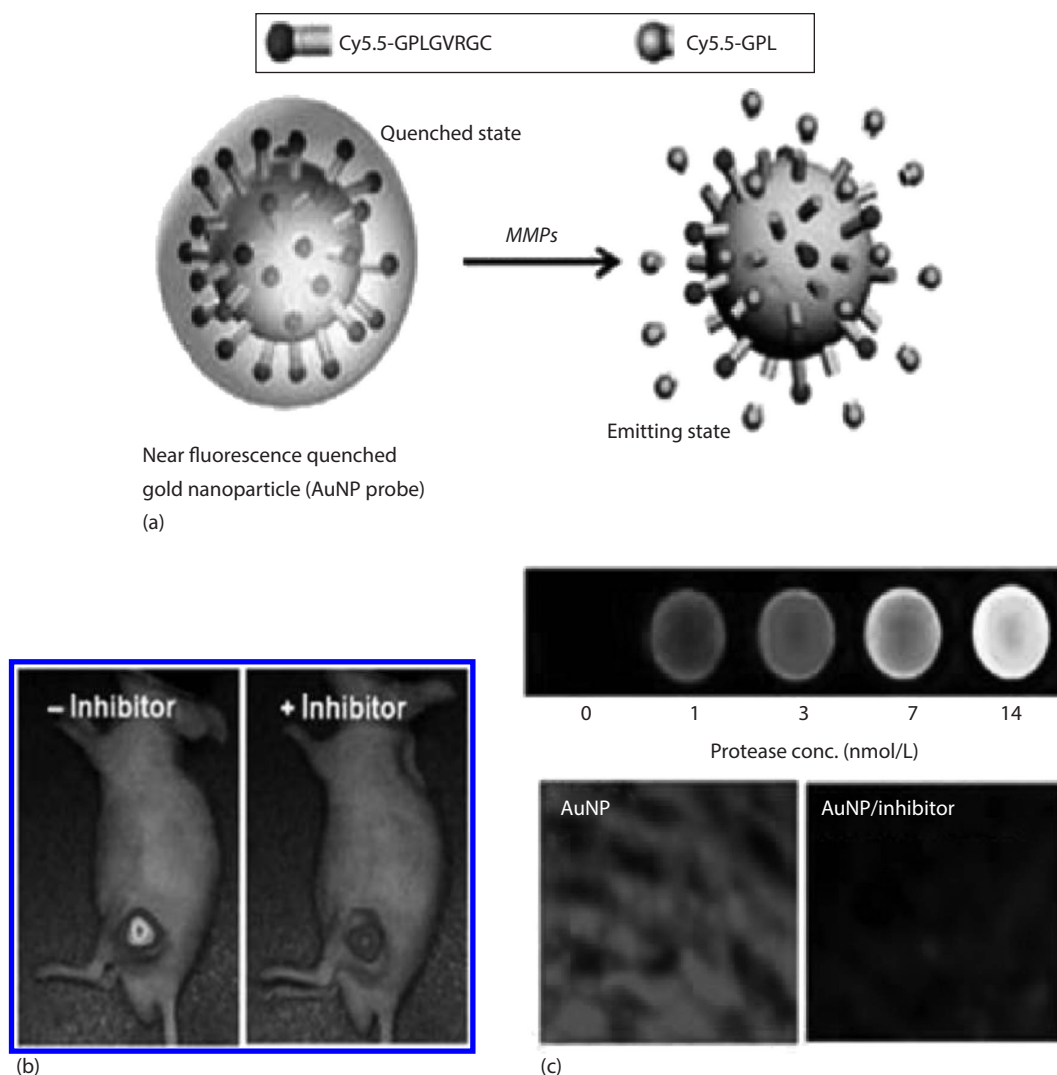
Figure 14.21 The AuNP-quenched activatable probes for in vitro protease detection and in vivo cancer imaging. (a) Schematic diagram of gold-quenched MMPs-activatable probe. (b) In vivo near-infrared imaging of MMPs-positive SCC7 tumor bearing mice after intratumoral injection of probes without (left) or with (right) MMPs inhibitor. (c) Optical fluorescence image containing the probe in the presence of various concentrations of MMP-2. (d) Fluorescence microscopy of excised SCC7 tumors. (Modified from Lee, S. et al., *Angew. Chem. Int. Ed. Engl.*, 47(15), 2804, 2008. With permission.)

of a highly quenched state. These have led to the development of a number of AuNp based imaging probes. In Figure 14.21, Lee et al. reported AuNP-quenched activatable probes for use in in vitro protease detection and in vivo cancer imaging.[101] The construct was based on an MMP-2 sensitive peptide sequence conjugation Cy5.5 a fluorescence dye and AuNP. The quenching of Cy5.5 was accomplished through NSET interactions with the gold nanoparticles as well self-quenching through FRET. Incubation with the enzyme MMP2 under in vitro conditions caused the cleavage of the peptide leading to fluorescence recovery. The probe showed similar results under in vivo conditions in mice bearing MMP2 positive SCC7 tumors. Carbon nanotubes have also found use

as potential optical imaging agents. Robinson et al. was one of the first to report the use of carbon nanotubes as potential dual imaging as well as therapeutic agent for cancer imaging and therapy.[102] SWCNT distribution within tumors was tracked at a high spatial resolution due to their intrinsic optical properties. This was one of the first reported uses of carbon nanotubes as candidates for image guided photothermal treatment of cancer.[103–106] Magnetic nanoparticles functionalized with fluorophore Cy5.5 and loaded with siRNA have allowed for sensitive detection of siRNA delivery in animal models with both optical as well as MR imaging. The MR imaging also allowed for monitoring of long term therapeutic efficacy following injection of the nanoparticles.[107–111] Other nanoparticle platforms such as polymeric nanoparticles, silica nanoparticles, liposomal formulations, have all been reported for preclinical optical imaging of cancer, CNS, inflammation, cardiac diseases and other disease states.

Preclinical Imaging of CNS Disease

Neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD), are becoming an increasingly urgent public health concern, particularly among aging populations. PET and SPECT imaging of brain functions help illustration on mechanism and progress of AD and PD in molecular level.

Parkinson disease (PD) is associated with nigral degeneration and striatal dopamine deficiency and is not known to occur in any species other than humans. Therefore, the most widely used animal model for this disease is the toxicity induced in the nigrostriatal pathway of C57BL6 mice following the administration of 1-methyl-4–1,2,3,6-tetrahydropyridine (MPTP). The ^{18}F-FDG PET reveals reduced lentiform nucleus glucose metabolism. Non-invasive functional imaging of dopaminergic change in the striatum was assessed by using positron emission tomography (PET) to visualize and quantify the uptake of ^{18}F-dopamine in brain of PD animal model.[112]

Showing striatal dopamine terminal dysfunction with PET supports the diagnosis and rationalizes the use of dopaminergic medications (see Figure 14.22). The PET imaging can detect changes in striatal dopamine levels after ^{18}F-dopamine administration and correlate these functional changes to motor responses.
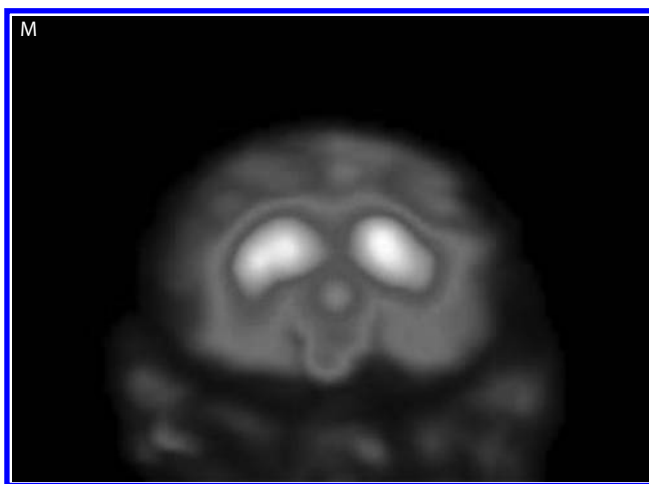
Figure 14.22   Functional brain imaging. 2.5 mCi of ^{123}IMZ, a DA transporter probe, was I.V. injected in a normal rat imaged at 3 h post-injection time point by a NanoSPECT/CT scanner. (Image provided by MPI Research Inc., Mattawan, MI.)

The neurobiologic processes support the use of metabolic imaging technique, namely [18]FDG-PET imaging, in the study of Alzheimer's disease as brain perfusion imaging. Most recently, amyloid imaging has been used in studies seeking to elucidate the natural history of Alzheimer's disease, early detection, and monitoring the treatment of Alzheimer's disease.[113–115]

One of applications of optical imaging includes cell tracking following transplantation in cases of neurological disorders and stroke by use of bioluminescence. Kim et al. used BLI to track murine C17.2 NPCs after transplantation in a murine model of stroke.[116] Their aim was to follow migration, but also to study possible proliferation and quantification of the transplanted cells. It was found that the maximum photon emission was observed at the site of experimental injury. The signal was maintained for 7 days following injection of the cells. Migration was observed to the ischemic site following injection. This migration was not seen in healthy animals indicating clearance of the cells from the animals. QDs have also been used for stem cell trafficking with good success. Kawabori et al. have demonstrated the use of QDs with NIR fluorescence for in vivo tracking of cells within the brain.[117] Using a permanent model of middle cerebral arterial occlusion in rats, the authors tested the potential of bone marrow stromal cells for functional recovery when transplantation occur 7 days post stroke via two different delivery routes. Intracerebral transplantation of cells was found to be the best mode of transplantation as fluorescence was detected for 7 days following transplantation. No fluorescence was observed following intravenous transplantation. The fluorescent probes mentioned above need to be excited by an external light source, which makes the excitation of deeply transplanted cells difficult. Two-photon or multiphoton excitation techniques have been proposed in the literature to overcome this issue. Chronic neurodegenerative diseases have also been imaged at a preclinical level using fluorescent agents. Recent work from Stanimirovic and Zhang demonstrate the use of labeled beta amyloid peptides to study transport across the blood brain barrier.[117] Beta amyloid peptides were labeled with Cy5.5 and used for imaging transport in normal as well as P-glycoprotein/ABCB1 and BCRP/ABCG2 pump deficient mice. The increased accumulation of beta amyloid peptides is correlated to Alzheimer's patients and elder non-demented patients. Such studies could be useful in evaluating kinetics of brain elimination of intra-cerebrally-injected compounds for a variety of diseases.

Preclinical Imaging of Autoimmune Disease

Autoimmune diseases are a heterogeneous class of diseases characterized by chronic inflammation of the target organ and often requiring lifelong treatment. One of the most important progress in the study of autoimmune diseases is the development of modern molecular imaging techniques by the production of specific radioactive probe which contributes to the identification of immune process responsible for various autoimmune diseases, such as rheumatoid arthritis (RA), age-related muscular degeneration (AMD), Type 1 diabetes Mellitus (IDD), and Crohn disease. Here we use an imaging study on initiation and progression of rheumatoid arthritis in a mouse model (see Figure 14.23).[12] These imaging studies described a novel detection system for determining the localization patterns of arthritogenic anti-GPI IgG in the joints of normal healthy mice, using rodent-scale positron emission tomography (microPET). The microPET R4 scanner permits dynamic noninvasive high resolution imaging of radiolabeled GPI-specific IgG in mice at multiple timepoints. The dynamic PET imaging results illustrated that anti-GPI IgG rapidly localized within minutes to distal joints of the front and rear limbs and remained there for at least 24 hours. These kinetics data were consistent with a mechanism of direct antibody recognition of GPI in the joints to trigger the rheumatoid arthritis in animal model.[13]

The fluorescence imaging can be used for monitoring immune responses and demonstrating the tissue specificity of the construct in laboratory animal models. A similar approach was used for detection of reactive oxygen species (ROS) found commonly in patients with rheumatoid arthritis and malignant tumors. Under these conditions there is also overexpression of hyaluronidase which
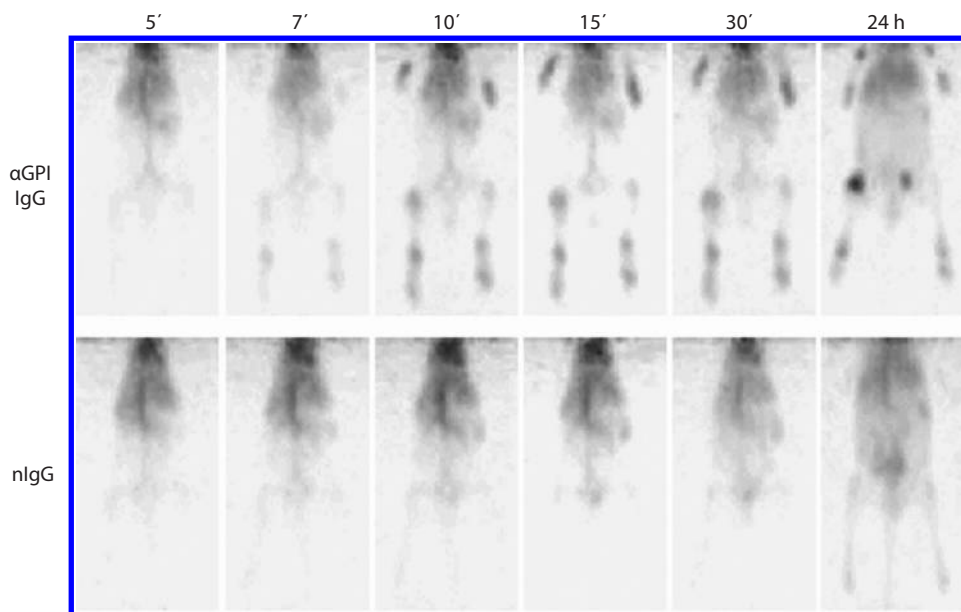
Figure 14.23 Initiation of rheumatoid arthritis with a rapid localization of ^{64}Cu-anti-GPI IgG to the distal joints vs. normal IgG distribution. (Images adapted and modified from Wipke, B. T. et al., *Nat. Immunol.*, 3, 366, 2002.)

degrades hyaluronic acid (HA).[118] Park et al. have demonstrated a probe to measure the ROS genera-tion by conjugating an NIR fluorophore to a thiolated HA which can be attached to AuNPs.[119–120] The newly synthesized nanoparticle allowed for ROS detection in laboratory animal models of rheumatoid arthritis and metastatic ovarian cancer.[121] The signal intensity derived from the abnor-mal tissues was significantly higher than other systemic organs.

Imaging Animal Model of Infectious Disease

Infectious diseases are the second leading cause of death worldwide. As age progresses, the immune system undergoes numerous changes that may affect our susceptibility to infection. Laboratory-animal imaging has become an important research tool in studies of infectious diseases and has significantly contributed to both our understanding of pathogenesis and preclinical investi-gations on drug development.

The mouse model of *infectious disease* has been extensively investigated worldwide. An impor-tant caveat to the epidemiological studies is that they usually combine several different routes of infection. Some studies have indicated that the route of infection with *Francisella* is an important determinant of bacterial dissemination as well as disease progression and outcome. In a PET study, ^{64}Cu(II)-PTSM, a tracer for detecting hypoxia, was used to radiolabel *Francisella tularensis* and to evaluate the bacteria trafficking of different routes in mice. The PET imaging study shows the dis-semination of *F. tularensis*, the cause of tularemia, when administered intranasally (i.n.), intratra-cheally (i.t.), intragastrically (i.g.), intradermally (i.d.), intraperitoneally (i.p.), or intravenously (i.v.) in mice.[10] The results demonstrated that *Francisella* rapidly disseminates within hours to multiple tissues via most routes of administration, although different trafficking patterns were observed. Infection via the pulmonary routes resulted in rapid spread to the lung and gastrointestinal tract (see Figure 14.24). In fact, this direct radiolabeling and imaging strategy can be used for study of
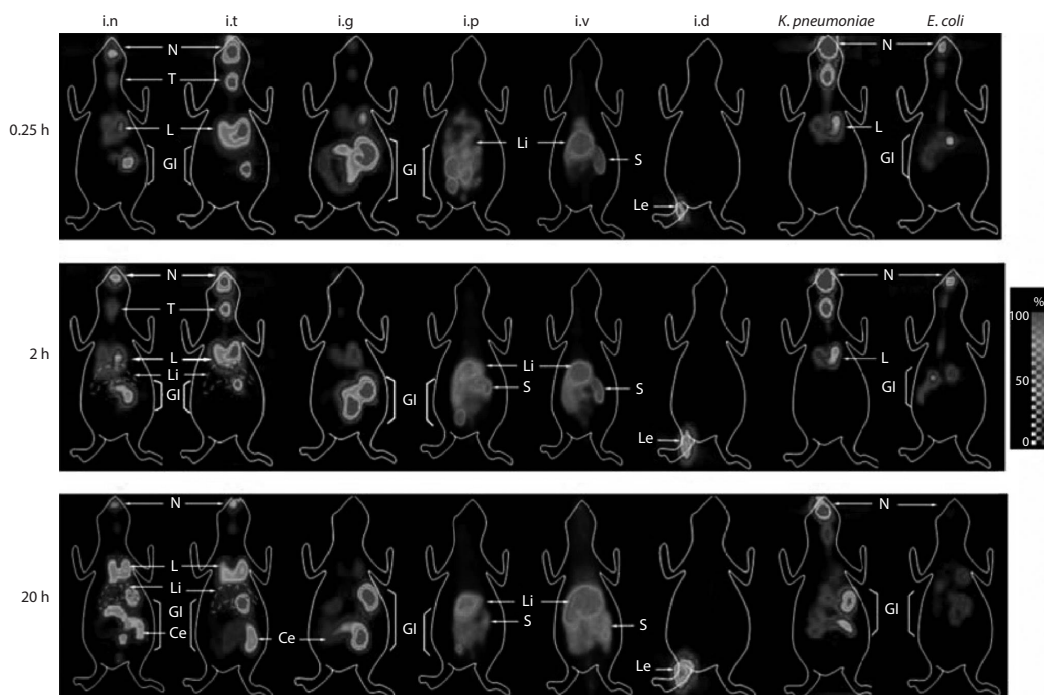
Figure 14.24 MicroPET imaging of bacterial infection of mice. Two-dimensional images of representative mice (ventral view) are depicted in the figure. MicroPET images were recorded over time from mice infected with either ^{64}Cu labeled *F. tularensis* subsp. *novicida*, ^{64}Cu labeled *Klebsiella pneumoniae* or ^{64}Cu labeled *Escherichia coli*. Mice were infected intranasally (i.n.), intratracheally (i.t.), intragastrically (i.g.), intradermally (i.d.), intraperitoneally (i.p.), or intravenously (i.v.) with *F. tularensis* subsp. *novicida* (2×10^9 CFU/20 μL) and i.n. with *K. pneumoniae* and *E. coli* using the same infection dose. Images were obtained at 0.25, 2 and 20 h post-injection. The punctuated line represents the liver. The scale on the right hand side of the figure is a linear scale that indicates percentage with light indicating 100% of signal and dark 0%–10% or the lowest amount detected by imaging. Abbreviations: N: Nasal cavity, T: Trachea, L: Lung, GI: Gastrointestinal Tract, Li: Liver, S: Spleen, Le: Leg, Ce: Cecum. (Images adapted and modified from Ojeda, S. et al., *BMC Microbiol.,* 8, 215, 2008.)

various laboratory animals of infectious diseases since the noninvasive imaging techniques permit enhanced information through longitudinal studies of the same animal during the infection process. The future development of multimodality molecular imaging studies for investigating the pathogenesis of infection will provide researchers multiple tools to have a positive impact on treatment of infectious diseases.

Preclinical Imaging of Cardiac Disease

Imaging-based approach on noninvasive evaluation of myocardial function plays more and more important role for preclinical imaging research and clinical practice, such as myocardial metabolism,[122] congestive heart failure, atherosclerosis, thrombosis, and stem cell therapy in myocardial infarction.[123] PET and SPECT/CT imaging is most effective if motion artifacts can be filtered out. This is typically performed by using physiological monitoring equipment to introduce "gating" tags to the raw acquisition data, indicating the start of the cardiac and respiratory cycles by [201]TI and ^{99m}Tc labeled radioactive probes (see Figure 14.25).
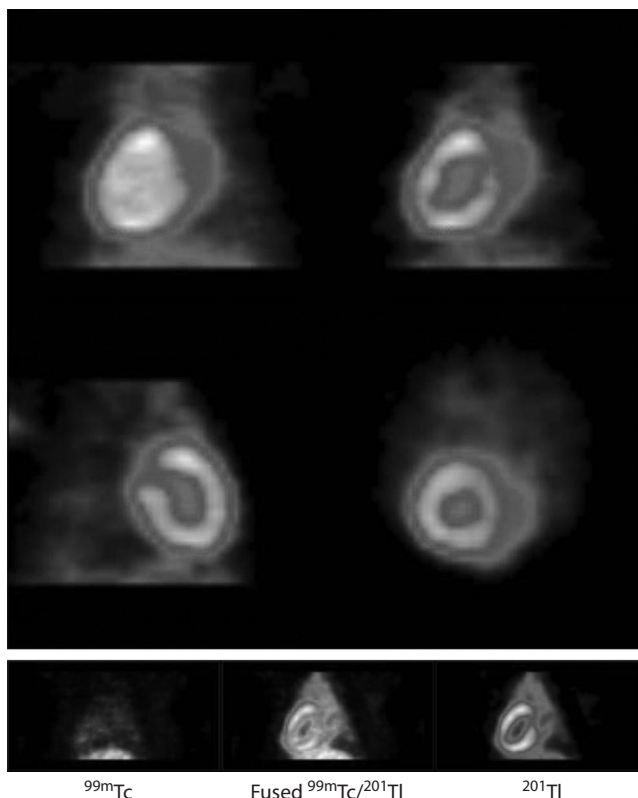
Figure 14.25   Cardiac gated imaging by SPECT. Rat was injected with 1.5mCi ^{99m}Tc and 2.5 mCi ^{201}TI with a simultaneous imaging of multiple radiolabeled compounds using different isotopes. (Image provided by MPI Research Inc., Mattawan, MI.)

In the use of a combination of bioluminescent cells and fluorescent probes, Weissleder et al. have been able to image the beating of heart cells at a cellular level in live mice (see Figure 14.26). Combined with the intravital microscopy and multiphoton imaging, the authors demonstrate the in vivo optical sectioning and dual-channel time-lapse fluorescence imaging of cardiac ischemia.[124] The NIRF imaging has also been used for imaging of plaques in coronary arteries. Weissleder and colleagues have developed an NIRF catheter for intravascular imaging of protease activity.[125] The catheter used for the study was of clinical grade and allowed for efficient visualization of plaques in a rabbit model using a commercially available NIRF agent called Prosense®. This study allows for the theranostic use of NIRF imaging of plaques in high risk patients, thus potentially reducing incidences of acute coronary syndrome with interventional pharmaceutical or mechanical interventions.

Optical imaging technology has also played an important role in the risk minimization and improved clinical translation. The idea is to select fluorescent molecules that will allow for the maximum possibility of long term clinical success. This will allow for accelerated use of fluorescence based methodologies in humans. Besides camera-based fluorescence imaging methods, advanced photonic approaches including multispectral optoacoustic tomography[126] or fiber-based confocal methods can further improve the theranostic and diagnostic potential of optical molecular imaging in laboratory animal models of human cardiovascular diseases and thus translate to future clinical practice.
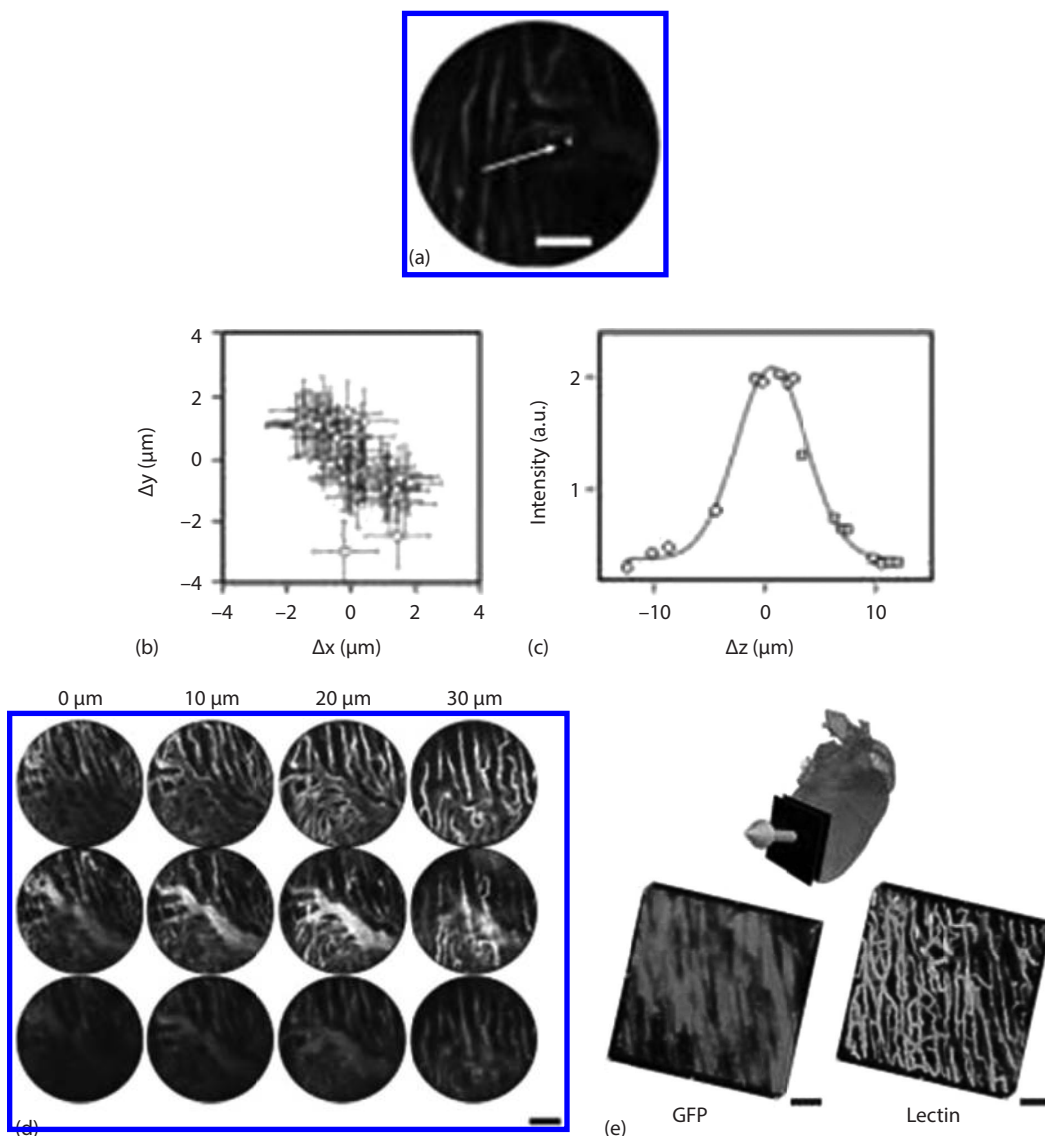
Figure 14.26 Image the beating of heart cells at a cellular level in live mice. (a) A stabilized image of a fluorescein isothiocyanate (FITC)-labeled bead (diameter: 5 μm), trapped within a microvessel, used to determine the overall stabilized imaging resolution. Scale bar, 50 μm. (b) The planar position coordinates of the trapped bead are plotted at different time points to allow acquisition of consecutive unprocessed images. Error bars correspond to error in position determination. A resolution of 4 μm was achieved with hardware stabilization. Gating improved the resolution to 2 μm. (c) Characterization of the axial resolution achieved using the gating modality in combination with the motion compensation stabilizer. Here, the axial position of the bead within the imaging time gating window was determined at different depths. (d) In vivo optical sectioning of the myocardium. Top row, lectin. Mid row, Angiosense-680. Bottom row, fusion of top and mid row images. Images were taken at different depths in 10 μm increments, using a 20× MicroProbe objective. Scale bar, 50 μm. (e) Optical sectioning along the indicated direction (arrow) allowed three-dimensional reconstructions in a GFP-expressing mouse, with GFP-expressing myocytes and lectin differentiated. Scale bars, 50 μm. *(Continued)*

(f)

CONCLUSION AND FUTURE PERSPECTIVES

The discovery of drugs and their development rely on the scientific understanding of biology and pathology at molecular, cellular, and organism level *in vivo*. The various modalities of molecular imaging are quickly demonstrating their value to many investigators in both preclinical development in laboratory animals[127] and human clinical practice.[128]

Molecular imaging is rapidly evolving. Numerous molecular and cellular events are being visualized from a living organism at the cellular level to laboratory animal models and translating to a variety of human diseases. The existing technologies have allowed us to image genetic phenotypes, protein-protein interactions, and signal transduction pathways noninvasively to understand the initiation and progression mechanism of various human diseases through laboratory animal models.

Multiple imaging agents and multimodality imaging platforms designed to take advantage of the basic physical elements of each can be used to provide insights into the physiological mechanisms of action and basic biological function of targets under drug development process, whether they are small molecules or biologic macromolecules.[129] Care should be taken when selecting the appropriate imaging modality for each biological target. Special consideration should be given to the potential impact on the native function of test article when imaging requirement includes the binding of an imaging probe to the test article for functional imaging *in vivo*. By understanding the strengths and limitations of each imaging platform, one can use them together, known as a multi-modality imaging process, to maximize the understanding of concurrent morphologic and functional changes in living laboratory animals.

The use of imaging techniques in laboratory animals will also lead to further insights into the molecular pathology of animal models of human diseases, as well as to the development of new molecular-targeted drugs *in vivo.* The incorporation and use of laboratory animals in appropriate imaging platforms are becoming better understood, with respect to both time and expense, during preclinical drug discovery and drug development process.

With a sturdy foundation of experimental preclinical imaging in laboratory animals, one can move into clinical development for molecular imaging allowing a new "precision pharmacology" that can have an important role in drug development. In the use of imaging experimental medicine will add complexity to the planning of clinical development programs and increase the cost per patient studied. However, these well-designed studies will answer key questions earlier and with smaller numbers of subjects for more confident decision-making. In the future, applications of molecular imaging to the development of drugs can add further value with their translation to clinical use as a companion diagnostic for patient stratification, staging, segregation, disease recrudescence, adaptive clinical trial work and enabling in commercial clinical applications of higher patient efficacy and lower overall healthcare cost.

ACKNOWLEDGMENT

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Genetically Modified Animal Models

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CONTENTS

INTRODUCTION

Genetically modified animals (GMA) have been developed to produce animal models of human disease and for research, to produce therapeutic or industrial products, to enhance animal food production, to improve disease resistance and animal health, and to enhance companion animals (FDACVM, 2009). Mice (*Mus muscularis*) are the most frequently used GMA in toxicology but this review will also reference the uses and issues associated with other GMA in toxicology. The first GMA, transgenic mice, were first reported (Gordon and Ruddle, 1981) and then shown to express activity of

exogenous genes (Brinster et al., 1981) more than 30 years ago. Stable genetic modifications have now been extended to most mammals including nonhuman primates (*Macacca* spp.) and non-mammals (Gama Sosa et al., 2010). Induced genetic modification of rodents and nonrodents are useful tools for research and development of new therapeutics, production of therapeutics and human gene therapy. Of these models, genetically modified mice (GMM) have the longest history of use in research and development (pharmacology, metabolism, and efficacy models), safety and reproductive toxicology and carcinogenicity testing. Genetic alterations pioneered in mice have been less successful in rats (*Rattus norvegicus*) but an increasing number of transgenic and knock down rats have been developed over the last decade. Genetic manipulation of other toxicologically relevant species is considerably more difficult than manipulations in rodents, and use of genetically manipulated rabbits (*Oryctolagus cuniculus*) (Murakami and Kobayashi, 2012), nonhuman primates (Chen et al., 2012), dogs (*Canis familiaris*) (Hong et al., 2009) and pigs (*Sus scrofa*) (Luo et al., 2012) are in early development and likely to be restricted to in vivo pharmacology and efficacy evaluations for the near future.

The three general categories of mutation models are reverse and forward genetics, and humanized models (Table 15.1). GMA, predominantly GMM are created using a variety of techniques via reverse genetics (gene is altered first) (Gama Sosa et al., 2010; Zheng et al., 2012). Spontaneous and *N*-ethyl-*N*-nitrosourea (ENU)-induced mutations (Russ et al., 2002; Zheng et al., 2012) are identified by forward genetics (phenotype identified first), and "humanization" of animal models with human genes or tissues (Shultz et al., 2007; Hasegawa et al., 2011).

Transgenic is a term often generically used to refer to GMA with transpecies genetic alterations. More precisely, transgenic refers to random insertion of genetic modifications, from the same or a different species, that result in random amounts and location of the transgene expression. Conversely, in gene targeting (knockout and knock-in) the wild type gene is specifically replaced or exchanged by homologous recombination using mouse embryonic stem (ES) cells with the desired mutation injected into blastocysts to produce chimeric mice. Chimeras with the stable mutation are bred to produce the founder lines. Depending on the construct, knockout procedures may result in defects in both alleles (homozygous), loss of an allele (hemizygous) or a defect in a single allele (heterozygous) and subsequent complete (null) or partial loss of expression of the gene product through deletions, point mutations, inversions or translocations. Gene trapping is a high through put method for creating knockout mice, using a reportable insertion sequence that prematurely inactivates transcription in ES cells. Unlike the randomness of transgenic construction, knock-in models target the gene with precise modifications (amounts and location) down to a single nucleotide. Knock down frequently uses RNA interference with microRNA (miRNA) or small interfering RNA (siRNA) to suppress gene transcription and protein production. This technique has proven successful in nullifying protein production in rats. To avoid lethality in embryos and neonates, or for temporal, tissue or cell specific gene expression, transgenic and gene targeting models can be inducible or conditional using modifications such as Cre/loxP and tet regulatory systems. GMM models can also be created using retroviral-mediated gene transfer, somatic cell mutagenesis/nuclear transfer, injection of zinc-finger nucleases or multiple combinations of all of the above techniques. A newer tool with broad applicability to genetic modification in mice and rats, and potentially gene therapy in humans, are the "Sleeping Beauty" (SB) and "PiggyBac" (PB) transposon systems (Largaespada, 2009). Class II DNA transposable elements (transposons) are synthetic non-viral gene delivery vectors that are mobile through cut-and-paste transposition with the genone. Transposons occur widely in nature, but are normally inactive in vertebrates. The SB reconstructed from the salmon genome, and PB from insects are active vectors that operate in mice and rats. After cloning the modification into the transposon vector, a transposase encoded by the transposon, can excise and re-integrate the transposon into the genome of cell lines and whole organisms. Transposons have been most successfully used in research to create custom models of cancer (Howell, 2012), but are also being used to produce highly GMA with specific single or multiple mutations that may eventually find their way into toxicology testing. The first clinical trials using a genetically engineered SB transposon for B-cell malignancies have been initiated (Hackett et al., 2010).

Table 15.1 Types of Genetically Modified Models in Toxicology

Type	Genetic Manipulation	Comment
Reverse Genetics (Gene Altered First)		
Transgenic	Random insertion of new genes from the same or another species or overexpression of endogenous genes in natural or ectopic locations.	Generally results in gain of function but can also result in loss of function.
Knock-in	Targeted exchange of a mutated DNA sequence for the endogenous sequence at a specific locus.	Controlled insertion, generally only at expected loci and in expected cell types or locations allows evaluation of specific mutations.
Knockout	Targeted replacement or disruption of coding exons with a drug selection marker to disrupt one (heterozygous) or both (homozygous) alleles and produce null, truncated or non-functioning proteins.	Can result in a deletion, point, inversion or translocation mutations. Used to evaluate the effects of loss of genes and model disease.
Knock down	Uses RNA interference with endogenous or exogenous (generally viral) siRNA or endogenous miRNA to cleave mRNA and silence the gene or repress RNA translation to deplete gene products.	Protein expression reduced or eliminated.
Inducible or Conditional	Cre-lox or other technology used to insert, knockout or rearrange DNA with temporal and location control over expression.	Can be used to control the genetic alteration spatially and temporally or intermittently, and to circumvent embryolethal mutations.
DNA Transposon Systems	Genetic modification cloned into transposon system vectors ("Sleeping Beauty" and "Piggybac" most frequently) and transposase will stably cut-and-paste the vector into the genome.	Results in specific and efficient localization. Can be used for gain of function or loss of function mutations, conditional mutations and complex mutations.
Forward Genetics (Phenotype Identified First)		
Spontaneous	Genetic mutations that arise spontaneously within breeding colonies.	Morphological or behavioral changes used to identify altered genes.
N-ethyl-N-nitrosourea (ENU) mutagenesis	Large scale screening programs using chemically-induced point mutations.	Morphological or behavioral change used to identify altered genes.
Humanized Mice		
Genomically humanized	Human genes (often whole loci) replace murine counterpart.	See transgenic and knock in.
Chimeric reconstitution	Chimeric mice produced after reconstitution with human cells or tissues, by engrafting replacement human cells into genetically deficient mice (knockout or spontaneous mutation), genetic insertion that allows nontoxic removal of targeted murine cells, or toxic destruction of cells or tissues.	Often used to create mice with a functional human immune system or metabolism.

Forward genetics, using phenotypic analysis to identify molecular changes, still plays a role in drug discovery and target validation. Mice with spontaneous mutations can be used alone or further manipulated with additional induced genetic alterations to modify phenotypes similar to transgenic or gene targeting mutations. Large-scale programs using chemically-induced point mutations with ENU (Russ et al., 2002; Zheng et al., 2012) use morphological and behavioral changes in rodents to rapidly screen for therapeutic targets and to model human disease.

GMM are also valuable models when "humanized" to circumvent dissimilarities in molecules and functional pathways between mice and humans. While mice carrying human genes can be

considered humanized, most often humanized mice refer to chimeras that carry partial or complete human physiologic systems. Techniques for creating these models include reconstitution of human cells or tissues into mice with a spontaneous or induced deficiency (Shultz et al., 2007), nontoxic ablation of cells in mice carrying a drug-susceptible transgene (Boverhof et al., 2011; Hasegawa et al., 2011), toxic removal of cells or tissues with chemicals, surgical replacement of murine tissues with portions of human cells or tissues or a combination of these techniques (Suemizu et al., 2008). These chimeric mice are suitable research models for reconstitution of the immune system, and studies of autoimmunity, transplantation, infectious diseases, and vaccine development (Shultz et al., 2012), but are also finding a role in in vivo pharmacology and drug metabolism (Cheung and Gonzalez, 2008), particularly through humanization of liver in mice.

All of the above mutations cause gain-in-function or loss-in-function, generally with altered or variable lose in protein expression that may result in mice with a phenotype of (1) no physical or functional changes, (2) fetal lethality and abnormal structural or functional development of the viable fetus, (3) abnormal development or functional changes in postnatal cells and tissues, (4) functional changes in molecular or cellular signaling that may or may not produce a physiological or pathological change including onset and expression of tumor and non-tumorous lesions, and (5) behavioral changes. When well characterized, phenotypic and behavioral changes can be used to supplement or in some cases, supplant nonclinical toxicology studies in standard safety and efficacy programs. However, care must be taken in interpretation of results from any genetically altered animals used for toxicology testing. Typically, inbred mice with a 129 substrain, C57BL/6 substrain, BALB/c and FVB/N strains are used for generation of GMM. For gene targeting, ES cells of 129 substrains are inserted into other strains, often C57BL/6 substrains, to create hybrids that require at least 10 backcrosses to move the modification >99% onto a single genetic background. If not adequately backcrossed, undesirable spontaneous pathology from 129 substrains and unstable and pseudo-phenotypes are likely (Simpson et al., 1997; Wong, 2002). C57BL/6-derived ES cells are being used more frequently but the same issue arises when another mouse stain is the desired genetic background (Hayward et al., 2011). Substrain, gender, colony environment (including endogenous enteric flora), concurrent disease (endogenous retroviruses and subclinical and clinical infections) are all modifiers of, or can be the cause of, phenotypic, immunologic or behavioral differences in GMM (Barthold, 2004; Bothe et al., 2004; Sellers, 2012). These modifiers are also known to affect tumorgenicity in genetically modified carcinogenicity models (Donehower et al., 1995). For accuracy, the complete and standardized nomenclature for genetically modified rodent strains, including substrains, alleles and mutations, should be reported in toxicology studies using the reserved gene and allele symbols and if applicable, associated proteins. Standardized nomenclature and usage for mice and rats is available at the Mouse Genome Informatics (MGI) (Mouse Genome Database, 2013) and Rat Genome Database (RGD) (Rat Community Rat Nomenclature Guidelines, 2013) websites, respectively. This nomenclature has changed from that previously published, and differs from that used for human genes and proteins (HUGO Gene Nomenclature Committee, 2002). Gene nomenclature for all species can also be searched at the NCBI-NLM-NIH online database (National Center for Biotechnology Information, 2013).

GMM IN TOXICOLOGY PROGRAMS

GMM are currently used, or could be applied to any stage of discovery and development. There was quick acceptance of GMM as valuable tools in discovery, in vivo pharmacology, mechanistic and efficacy studies, and safety studies of biologics, monoclonal antibodies, vaccines and gene therapy, but not for Good Laboratory Practice (GLP) toxicology studies. Knock-in and humanized mice replacing the mouse gene with the human homolog most closely recapitulate the human system, but knockout, transgenic and conditional transgenic animals can also be suitable models for toxicology. Limitations on the use of GMM often include limited availability of the specific mutation on an

appropriate genetic background, and need for adequate lead time (often 1–3 years) to properly create or rederive, genotype, phenotype, functionally characterize and breed adequate numbers of mice for a safety or efficacy testing program (Bugelski et al., 2000). With knockout mutations characterization is often limited to homozygotes, although careful screening of heterozygous mutations may identify a phenotype of genetic haplosufficiency that is often a better representation of human disease or deficiency (White et al., 2013). Interpretation of the test results also needs to be carefully undertaken due to genetic and physiologic modifiers of phenotypes, previous incorrect or incomplete phenotype attribution, concurrent, acquired or opportunistic infections, lack of life-time historical data for most GMM (Bolon and Galbreath, 2002; Bussiere, 2008; Keane et al., 2011; White et al., 2013) and the possibility of mismatching GMM and their wild-type controls (Bourdi et al., 2011).

Short-term carcinogenicity testing in transgenic rodents as a substitute for the 2-year bioassay was slower to gain acceptance even after completion of an extensive and global validation program (Robinson and MacDonald, 2001) and acceptance of GMM in the ICH Guidance S1B (ICH, 1998). Although this guidance specifies rodents, only mouse models have been validated. Except for the p53 deficiency, comparable bioassay models in the rat are currently unavailable. While there is general regulatory acceptance of GMM in the United States, Canada, EU and Japan, use of alternative carcinogenicity models still need to be justified, and similar to unmodified animals, data interpretation must be undertaken carefully.

In Vivo Pharmacology and Efficacy Studies

There is an extensive body of literature documenting use of GMM for in vivo pharmacology and efficacy studies of biopharmaceuticals and small molecules in models of human disease. As these GMM models seldom fully recapitulate human disease states and genetic backgrounds affect the phenotype, care must be taken in selection of the model and in interpretation of results. In toxicology reports using GMM, a clear indication should be given of similarities and differences compared to the human disease, and interpretation restricted to statements of efficacy related to the specific elements of similarity instead of broad statements of efficacy. Where data exists, the known preclinical versus clinical correlates of these models (Zambrowicz and Sands, 2003; Politi and Pao, 2011; Singh et al., 2012b) should be provided in submissions. Recent literature reviews that define limitations of GMM models for specific diseases and organs should be consulted to learn strengths and weaknesses of models prior to their selection for preclinical studies. Examples of models for specific clinical indications include oncology (Weiss et al., 2002; Hansen and Khanna, 2004; Robles and Varticovski, 2008; Huse and Holland, 2009; Politi and Pao, 2011; Telang and Katdare, 2011; Roper and Hung, 2012), autoimmunity (Pizarro et al., 2000; Liang et al., 2006; Doetschman, 2011; Shultz et al., 2012), hemostasis and fibrinolysis (Car and Eng, 2001), obesity/metabolic syndrome/diabetes (Greiner et al., 2011; McMurray et al., 2012) and neuromuscular diseases (Howell et al., 1997, Vlamings et al., 2012; Sabbagh et al., 2013). The up-to-date status of available GMM models of disease are also accessible at the mouse and rat genomic websites (Phenotypes Alleles & Disease Models at MGI, 2013; Phenotypes & Models at RGD, 2013), respectively.

GMA, particularly those labeled with fluorescent markers (Boverhof et al., 2011; Murakami and Kobayashi, 2012) are valuable tools for pharmacological evaluation by in vivo imaging down to the cellular level. Mice with one or more deletions can also be used to fully understand potential tissue targets associated with therapeutic suppression of gene products (Mahler et al., 1996a) and to screen therapeutic strategies for specific diseases (Gil-Mohapel, 2012). Although the size of mice frequently limits pharmacokinetic (PK) sampling, GMM models can be used to obtain PK data to help in interpretation of study results. As the genetic backgrounds of GMM are generally not the same background used in safety toxicology studies, PK data in GMM can provide valuable information to bridge efficacy and safety studies. Additionally, PK evaluations conducted during in vivo pharmacology and efficacy studies can help to identify possible differences between normal and

diseased states. GMM can also be used to evaluate pharmacologic targets, receptor and transportation pathways, and metabolic differences (Burki and Ledermann, 1995; Cheung and Gonzalez, 2008; Politi and Pao, 2011). The multidrug carrier P-glycoprotein is encoded by the multidrug resistance (Mdr) gene within the ATP-binding cassette (ABC) transporter, which is an efflux pump related to Mdr. Abcb1a (Mdr1a) knockout or deficiency in mice, rats and dogs (van Asperen et al., 1996; van Waterschoot and Schinkel, 2011; Geyer and Janko, 2012) and double Abcb1a/1b knockout in mice (Doran et al., 2005) are examples of models for efflux transporter genes, overexpression of P-glycoproteins and blood brain barrier dysfunction. Deficiencies in these models allow studies of drugs on the brain, toxicity of chemicals utilizing cellular P-glycoproteins and multi-drug resistance in human tumors of chemotherapeutic failure.

Absorption, Distribution, Metabolism, Excretion, Pharmacokinetics and Drug–Drug Interactions

Genetic manipulations involving specific gene and allelic modifications and chimeric mice with humanized liver have been applied to discovery and early development phases, but have not been reported to replace typical absorption, distribution, metabolism and excretion (ADME) or pharmacokinetic profiling in safety studies. Drug metabolism and excretion, pharmacokinetics, metabolite evaluation, drug–drug interactions and drug resistance can be determined using GMM with specific metabolic profiles created by transgenic insertion of human genes, inactivation/deletion of cytochrome P450 and other xenobiotic-metabolizing enzyme genes and receptors (Ghanayem et al., 2000; Ariyoshi et al., 2001; Muruganandan and Sinal, 2008; Powley et al., 2009; Boverhof et al., 2011) and humanization of mice reconstituted with human hepatocytes (Muruganandan and Sinal, 2008; Peltz, 2013). While GMM with individual xenobiotic metabolizing enzymes or pathway components alterations can be useful, not all components of metabolism are available in GMM models, multiple component insertion or deletion models may be needed for mechanistic or safety studies, proper characterization of these models can be time-consuming and expense, and these GMM retain the mouse orthologues of metabolic pathways (Ghanayem et al., 2000). Consequently, mice humanized by reconstituted with human hepatocytes containing whole human metabolic pathways including Phase I and II enzymes and transport proteins are receiving more attention.

Immunodeficient mice overexpressing urokinase-type plasminogen activator (Plau, formerly uPA), a knockout of the fumarylacetoacetate hydrolase (Fah) gene and more recently, transgenic insertion of the thymidine kinase (TK1) gene are the most common humanized liver mouse models. In the Plau model, hepatocellular damage results in liver failure but on a severe combined immunodeficiency (SCID) or recombinant activating gene 2 (Rag2) deficient background, these mice can be reconstituted with human hepatocytes. Excess neonatal mortality, a bleeding diathesis and an inability to breed the homozygous individuals limit the use of this model. The Fah deletion in mice causes accumulation of a fumarylacetoacetate toxic metabolite. This metabolite is blocked by 2-(2-nitro-4-trifluoro-methylbenzoyl) 1, 3-cyclohexedione (NTBC) so that mice can be maintained without liver damage until withdrawal of NTBC, followed by repopulation with human hepatocytes (with normal FAH expression). In combination with immunodeficient Rag2 and interleukin 2 receptor common gamma chain null mice (FRG mouse model; $Fah^{-/-}/Rag2^{-/-}/IL2rg^{-/-}$), human hepatocellular repopulation is improved compared to Fah mice. Serial liver transplantation further improves repopulation and can be used to generate additional humanized mice from limited human liver xenografts (Strom et al., 2010). Fah mice are also susceptible to liver carcinogenesis and require drug treatment to suppress liver tumors (Nishimura et al., 2013; Peltz, 2013); Further refinement in liver metabolism models, and the most promising model (Peltz, 2013), involves insertion of a *Herpes simplex* thymidine kinase (UL23) vector into immunodeficient NOD/Shi-*scid* IL2Rg^{null} mice (TK-NOD). Administration of a nontoxic dose of gancyclovir (GCV) causes destruction of liver cells and xenografts are subsequently readily accepted. Histologic, molecular and functional

studies have indicated that TK-NOD mice results in a mature, functionally stable and long-lived human liver that does not require maintenance with drug treatments (Hasegawa et al., 2011; Hu et al., 2013). Doses of GCV administered after the initial human hepatocellular repopulation can also be used to remove additional residual mouse hepatocytes and maximize the human hepatocytes (Hasegawa et al., 2011).

Humanized mouse models generally demonstrate the 3-dimensional architecture, lobular enzyme distribution, gene expression profiles and human-specific drug metabolism pathways similar to mature human liver, but these models can also be repopulated with hepatocytes of a single or specific combinations of cytochrome P450 enzymes (Boverhof et al., 2011). To a lesser extent, phase II and I pathways can also be manipulated in these mice. Thus, humanized models are applicable to evaluation of hepatocellular metabolism and elimination, pharmacokinetics and enzyme expression and induction related to drugs, metabolites and drug–drug combinations, and to mechanistic studies of hepatotoxins (Strom et al., 2010; Hasegawa et al., 2011; Hu et al., 2013; Nishimura et al., 2013; Peltz, 2013). Less clear is how well these humanized models retain or recapitulate the structure and function of biliary, lymphatic, perisinusoidal and sinusoidal compartments and supporting endothelial, Kupffer and stellate cells, bile acid and pigment production, enterohepatic circulation, urea cycling, and hepatocellular production and metabolism of serum transaminases, carbohydrates, proteins, lipoproteins, lipids, or effects on other organs. Effects of residual mouse hepatocytes on human hepatocellular metabolism in these models have also not been well characterized.

Safety Pharmacology

While seldom used, mice and rats with spontaneous or induced genetic mutations could be useful as adjunct and mechanistic models in safety pharmacology studies. For instance, enhanced cardiovascular testing could be conducted in transgenic, knockout, knock-in and spontaneous mutation models of prolonged QT syndrome (Salama and London, 2007), respiratory safety tested in asthma models (Baron et al., 2012), and CNS assessments tested in models of Alzheimer's (Sabbagh et al., 2013) and amyotrophic lateral sclerosis (Mead et al., 2011).

Genotoxicity

Transgenic rodents with insertion of multiple copies the *Escherichia coli* markers *lacI*, *lacZ* and *gpt* delta have been accepted for chemical mutagenicity (OECD, 2011) in Europe and by the U.S. Environmental Protection Agency (U.S. EPA), but are generally only used as supplemental assays or for mechanistic genotoxicity studies (ICH, 2011a). Compared to in vitro testing, these transgenic systems have limited to no current commercial availability, are expensive, exhibit a relatively high spontaneous mutation rate and may be insensitive to clastogens, but they are superior for their ability to test mutations in multiple organs, and ability to use the same rodent strain in mutagenicity studies that will be used in carcinogenicity studies (Bitsch et al., 2005a). Transgenic mice with bacteria mutation markers include *lacI ± c-II* Big Blue® mice (C57BL/6Tac-Trp53tm1Brd-Tg(lacI) N5) and rats (F344 homozygous/no official gene designation) and *lacZ ± c-II* bacteriophage Muta™Mouse ([BALB/c × DBA/2]CD2F1 strain; Covance, Harrogate, England). These models are constructed with insertion of a target gene for the mutations and a shuttle vector to recover the target DNA from tissues. After treatment to induce DNA point mutations or deletions, one or more tissues are collected for DNA extraction, transferred to bacteria using phages and plated to agar with the readout as positive blue plaques or low temperature selection for Lambda *c-II* plaques. Chemicals validated in these transgenic mice have mutagenicity rates comparable to the mouse or rat bone marrow micronucleus (Dean et al., 1999; Bitsch et al., 2005a), mouse spot (Bitsch et al., 2005b) and rat liver unscheduled DNA synthesis (Dean et al., 1999) assays. Mice with the *lacZ* plasmid shuttle vector (C57BL/6-Tg(LacZpl)60Vij/J; The Jackson Laboratory, Bar Harbor, ME) also use a

positive selection process for color mutants (Dollé et al., 1999). In the Gpt delta mice (C57BL/6-TgN(gpt Delta))(Masumura et al., 2000) and rats (F344-NSlc-Tg [Gpt Delta]) (Matsushita et al., 2013) point and deletion mutations are differentially identified by 6-thioguanine and Spi− selection assays (Nohmi et al., 1996), respectively. The Spi− selection system and the *lac*Z plasmid mouse are the only assays capable of detecting large deletions (Nohmi et al., 1996; Masumura et al., 2000). The Gpt delta rat has been used as a short-term liver bioassay for tumor promotion (Matsushita et al., 2013). Mice with a deletion in DNA repair enzymes including adenine phosphoribosyltransferase (Aprt; several strain designations, hypoxanthine phosphoribosyltransferase (Hprt[b-m3]) and Xpa^{−/−} mice (C57Bl/6J-TgH(XPAIm)55Cmg) can also be used in mutagenicity assays or as an alternative to 2-year bioassays, but at least for the Aprt^{−/−} mice, their use should be restricted to mechanistic studies as these mice exhibit phenotypic and behavioral abnormalities (Van Sloun et al., 1998).

Genotoxicity for biopharmaceuticals can also be conducted using specific deletional or transgenic GMM. Although reported rarely, the mouse micronucleus assay has been used after treatment of a primatized™ monoclonal antibody of CD4 in a human CD4 transgenic mouse (Bugelski et al., 2000).

Biologics, Gene Therapy, Vaccines and Immunotoxicity

Uniquely, GMM are an accepted alternative for safety evaluation and risk assessment for biotechnology products. In the absence of a relevant species due to immunogenicity or lack of pharmacological activity, the ICH S6(R1) supports animal models of human disease, genetically modified models and surrogate molecules as alternatives for safety testing (ICH, 2011b). This includes transgenic or knock-in animals expressing a functional version of the human target, appropriate deletional models to evaluate target inhibition or replacement therapy, models of immunogenicity or complex (multiple) genetic modifications. Toxicology studies using GMM as alternative models have seldom been reported or may be difficult to identify if safety is reported in conjunction with predominately efficacy studies. A comprehensive report (toxicity, genotoxicity, immunotoxicity, host defense and reproductive toxicity) has been published for evaluation of a primatized anti-CD4 monoclonal antibody in huCD4 transgenic mice (Bugelski et al., 2000) and Aβ immunization in transgenic was used to support early Alzheimer's disease (Elder et al., 2010) clinical trials.

Gene therapy trials have frequently used induced murine mutation models to test efficacy, but when available, spontaneous mutation models in large animals are also valuable for efficacy and safety testing (Casal and Haskins, 2005). Dogs with spontaneous mutations have been used for preclinical proof of concept and safety studies in support of clinical trials for diseases such as Duchene muscular dystrophy (Howell et al., 1997) and Leber's congenital amaurosis (Narfström et al., 2003).

GMM have also been used in mechanistic studies to address post-marketing issues such as the potential of anti-angiogenic tumor therapy to enhance tumor invasion and metastases (Singh et al., 2012a). Mice reconstituted with a humanized immune system are also valuable models of immunity, immunotoxicology and drug development for vaccines, autoimmunity, transplantation (Macchiarini et al., 2005; Shultz et al., 2011, 2012), cytokine, chemokine and drug hypersensitivity reactions (Moser et al., 2001), hematotoxicity (Cai et al., 2011) and clinical indications such as diabetes (Greiner et al., 2011).

Developmental and Reproductive Toxicology (DART) and Juvenile Toxicology

DART studies conducted in GMM are accepted, and may be desirable or specifically requested by regulatory agencies for development of biologics (Buckley et al., 2008; Bussiere et al., 2009). Common findings in GMM such as low fertility and fecundity, embryolethality, peri-natal mortality and failure to thrive (runting) are major liabilities in DART studies, as these effects are often unexpected and may be the indirect or direct results of gene deletion or insertion (White et al., 2013). Issues with morbidity and mortality may be partially controllable through use of conditional mutations or rescue with temporary therapeutic supplementation (e.g., replacement of immunoregulatory products)

during critical periods, but phenotypic and functional abnormalities of these modified GMM need detailed characterization before use in safety studies (Bugelski et al., 2000). Other issues include the lack of contract research organizations that have experience with GMM in DART studies, lack of historical data, that phenotypic alterations in utero may not reflect the true pharmacologic activity postnatally, and pathway redundancy (Bussiere et al., 2009). DART studies have been reported in IL-12p40 knockout (Enright et al., 2011), IL-6 knockout (Sakurai et al., 2012) and developmental immunotoxicology for HuCD4 transgenic mice treated with an anti-CD4 monoclonal antibody (Bugelski et al., 2000; Herzyk et al., 2002).

There have been no reported studies that have specifically used juvenile GMM for GLP safety studies, but the same constrains that apply to the use of juvenile animals in any toxicity study would be of concern in using juvenile GMM in toxicology studies.

Photosafety Studies

Although supplemental photosafety studies in GMM with altered pathways of ultraviolet-induced oxidative damage, DNA repair and immunosuppression are possible, only cutaneous photocarcinogenicity models such as xeroderma pigmentosa group A ($Xpa^{-/-}$), $p53^{+/-}$ or $Xpa^{-/-}/p53^{+/-}$ double deficient models have been used with any frequency for altered DNA repair studies associated with ultraviolet (UV) light exposure. These GMM are viable models and are described below.

Short-Term Carcinogenicity Models

The expense of and inability of the combined mouse and rat 2-year bioassay models to consistently predict or correlate with human carcinogenicity risk have been well described (Jacobson-Kram et al., 2004). The complexity of the mechanisms of induction of and progression of tumor-induction is often dependent on genetic, epigenetic, environmental, age-related, toxin exposure and bioavailability, and confounding modifiers which cannot be adequately modeled in standard animal models of carcinogenesis. Additionally, the animals used to model carcinogenesis are generally inbred strains that have been selected over time for specific spontaneous genetic changes that limit or drive the spontaneous development of tumors and may involve mutations in other fundamental cancer-modifying pathways such as the immune system. Given the heterogeneity of the human population and species differences, current rodent models do not adequately recapitulate expected tumor and non-tumor risk in humans (Jacobson-Kram et al., 2004; Maronpot et al., 2004).

The International Life Sciences Institute/Health and Environmental Sciences Institute (ILSI/ HESI) initiative to evaluate alternatives to the 2-year bioassay (data compiled in entire volumes of *Toxicologic Pathology*, Vol. 26, 1998 and Vol. 29 Supplement, 2001), concluded that the neonatal mouse model (McClain et al., 2001) and in vitro Syrian hamster embryo (Mauthe et al., 2001) were of limited usefulness (Cohen, 2001). Up to 21 chemical compounds, but no biologics, were tested in 5 GMM models including the $p53^{+/-}$ knockout (Storer et al., 2001), Tg.AC (Eastin et al., 2001), rasH2 (Usui et al., 2001), XPA and $XPA^{-/-}/p53^{+/-}$ (van Kreijl et al., 2001) mice. Results for these models were mixed. The ILSI/HESI initiative provided evidence of the usefulness of all the tested GMM as alternatives to 2-year bioassays in mice, but testing of two GMM models by National Toxicology Program (NTP) supported use of the $p53^{+/-}$ and not the TG.AC model (Bucher, 1998). Overall, GMM models identified the majority of positive and negative carcinogens and had greater specificity than traditional 2-year bioassays, but were not predictive of tumor specificity for human organs and unable to provide information on mode of action or dose-responsiveness (Cohen, 2001; Jacobson-Kram et al., 2004). Limitations of these GMM models for carcinogenicity assays included false negatives for known human carcinogens (Prichard et al., 2003; Jacobson-Kram et al., 2004), limited ability to identify nongenotoxic carcinogens, and lack of data on lifetime tumor and non-tumor lesions for the strains used for these GMM (Maronpot et al., 2004). Use of GMM in

short-term assays in conjunction with a rat 2-year bioassay have been successfully used and data is accumulating for GMM models that is helping to better define and understand these alternative models in pharmaceutical and chemical carcinogenicity testing. The EU Evaluation, Authorization and Restriction of Chemicals (REACH) policy and the U.S. EPA, recognize the value of GMM but short-term bioassays in GMM are not specifically accepted in lieu of 2-year bioassays (Wells and Williams, 2009).

As with 2-year bioassays, GMM models are not entirely predictive of human carcinogenic risk, but faster screening, reduced study costs and reduction in animal numbers support use of selected GMM models as an alternative assay. Remarkably, substitution of these alternative models for the 2-year bioassay in pharmaceutical testing remains less than expected. The U.S. FDA has received several hundred proposals and fewer completed reports for pharmaceutical carcinogenicity testing in GMM, with a trend towards increasing use of the rasH2 over the p53$^{+/-}$ model, and with limited usage of Tg.AC and XPA$^{-/-}$/p53$^{+/-}$ models (Jacobson-Kram et al., 2004; Boverhof et al., 2011). In Europe, use of GMM models of carcinogenicity to support registration occurs infrequently (Friedrich and Olejniczak, 2011), possibly related to ethical considerations and lack of public support (Mepham et al., 1998). The regulatory need for carcinogenicity models continues to evolve and negative results for endocrine effects, genotoxicity and chronic study carcinogenicity factors in the rat, and use of a transgenic mouse assay might reduce the need to conduct any 2-year bioassays in rats in up to 40% of compounds (Sistare et al., 2011). As of yet, there has been no proposals to evaluate genetically modified rats, particularly the p53$^{+/-}$ rat as an alternative to the 2-year bioassay.

Along with the models tested in the ILSI/HESI initiative, other GMM have been investigated and continue to be tested (Table 15.2) by the NTP and and other organizations. General regulatory acceptance of GMM as alternatives for the 2-year mouse bioassay includes the heterozygous knockout of the Trp53 tumor suppressor gene (Trp53$^{+/-}$; p53$^{+/-}$) and two H-ras oncogene models (homozygous transgenic rasH2/c-Ha-ras2 and homozygous or heterozygous transgenic Tg.AC/v-Ha-ras2). The basis for these selected mutations is the frequency of human TP53 and murine Trp53 mutations or functional inactivation, and human HRAS and murine Hras protooncogene mutations in tumors (Gulezian et al., 2000). In an increasing number of cases, the rasH2 transgenic model is used as the alternative to the 2-year bioassay in mice, as this model can also evaluate nongenotoxic compounds. The p53$^{+/-}$ knockout, with or without knockout of Xpa$^{+/-}$, is restricted to use with genotoxic compounds. The transgenic Tg.AC homozygous $^{+/+}$ or heterozygous $^{+/-}$ models are limited to carcinogenicity testing of dermal compounds and is falling out of favor (Boverhof et al., 2011; Friedrich and Olejniczak, 2011; Nambiar and Morton, 2013). Until proven otherwise, other GMM models for carcinogenicity testing are most useful as mechanistic or complementary assays for genotoxicity and carcinogenicity, and are applicable to short-term carcinogenicity under limited testing circumstances. Other GMM models have been and continue to be evaluated as models of carcinogenicity but these are only applicable for specific tumor types. Transposon models of GMM and genetically modified rats have the potential to provide discrete and effective bioassay systems for carcinogenicity but these models have not be validated.

Transgenic CByB6F1-Tg(HRAS)2Jic Oncogene Model (Formerly rasH2)

This model is a hemizygous ($^{+/-}$) transgene with random insertion of the human HRAS (formerly c-Ha-ras) oncogene in addition to the endogenous murine Hras1 oncogene. Homozygosity (HRAS$^{+/+}$) is a lethal transgenic mutation in mice. The heterozygous mice (HRAS$^{+/-}$) have a low incidence of spontaneous tumors (alveolar bronchiolar tumors, vascular tumors, lymphoma and adenomas) by 6 months, but accelerated onset and malignancy of tumors after exposure to genotoxic and non-genotoxic carcinogens (Tamaoki, 2001; Urano et al., 2012; Paranjpe et al., 2013a).

Table 15.2   Overview of GMM Models Evaluated and Accepted or Selectively Useful as Alternatives for or Complementary to the 2-Year Bioassay

Common Name	Mutation	Nomenclature	Genotype	Predominate Spontaneous Tumor Phenotype	Comments
Generally accepted for short-term carcinogenicity studies					
Tg.HRAS$^{+/-}$ (Tg rasH2$^{+/-}$)	Insertion of human HRAS (c-Ha-ras) oncogene	CByB6F1-Tg(HRAS)2Jic	Hemizygous Transgenic	Alveolar bronchiolar tumors, hemangioma/sarcoma (multiple organs), lymphoma, squamous cell papilloma (skin, stomach), adenoma (liver, lung, Harderian gland), skeletal myopathy, vasculopathy and thymic abnormalities	Commonly used, acceptable for genotoxic and nongenotoxic compounds and implants
Trp53$^{+/-}$ (formerly p53$^{+/-}$)	Single allele inactivation of Trp53 tumor suppressor gene	B6.129-Trp53tm1BrdN5	Heterozygous Knockout	Lymphomas, sarcomas, confounding microchip transponder-induced sarcomas	N5 is partial congenic, N12 full C57BL6 congenic is available; suitable for genotoxic compounds; not suitable for implant testing
Tg.AC$^{+/-}$ or Tg.AC$^{+/+}$	Activated Hras (v-Ha-ras) oncogene homolog	FVB/NTac-Tg(Hba-x-v-Ha-ras)TG.ACLed	Homozygous or Hemizygous Transgenic	Odontogenic tumors, forestomach squamous papillomas, injury-induced epidermal squamous cell papillomas, lung adeno/carcinomas	Dermal carcinogenicity of genotoxic and non-genotoxic carcinogens; spontaneous tumors may not appear in wild type mice
Limited usage after validation testing					
Xpa$^{-/-}$	Xeroderma pigmentosum group A-like deficient nuclease excision repair gene	C57Bl/6J-TgH(XPAIm)55Cmg	Homozygous knockout	Cutaneous papillomas and squamous cell carcinomas, liver adenomas	Requires 9 months of exposure; carcinogen pathway specific
Trp53$^{+/-}$/Xpa$^{-/-}$	See above XPA and Trp53	C57Bl/6J-TgH(p53Im)Δ26 Cmg) x C57Bl/6J-TgH(XPAIm)55Cmg	Double knockout	Sarcomas (p53), adenomas (XPA), lymphomas (p53, XPA)	Requires 9 months of exposure; carcinogen pathway specific

Twenty ILSI/HESI studies confirmed carcinogenicity in CByB6F1-Tg(HRAS)2Jic mice after administrations of the majority of mutagenic carcinogens, a human hormonal carcinogen and liver adenoma induction in male mice with peroxisome proliferators, a non-genotoxic rodent carcinogen (putative noncarcinogen in humans) but no other non-genotoxic compounds (Usui et al., 2001). This model shows similar predictivity to known carcinogens in the 2-year bioassay, and is recommended for both non-genotoxic and genotoxic carcinogen short-term screening, although the mechanism for tumor induction is unknown (Tamaoki, 2001). Unlike the Trp53$^{+/-}$ model, the carcinogenic properties of medical implants can be successfully tested in the Tg.HRAS model (Palazzi and Kergozien-Framery, 2009). A recent retrospective analysis of short-term carcinogenicity assays using this model has shown that the spontaneous neoplastic changes in positive and negative controls are similar to those reported in the ILSI/HESI testing and indicate stability of this transgene for at least a decade (Nambiar et al., 2012). This same pharmaceutical group compared a series of 10 compounds in Tg.HRAS mouse models to 2-year rat bioassays and identified 2 nongenotoxic compounds that were positive in the short-term mouse and 2-year rat bioassay and considered a potential human risk. The 2-year rat bioassay was positive in an additional six rat bioassays with tumors considered false positives and irrelevant to risk assessment in man. Thus, Tg.HRAS mice were considered potentially more relevant to human carcinogenicity risk assessment (Nambiar and Morton, 2013) than the rat bioassay. The spontaneous nontumor pathology of Tg.HRAS mice from a large database has been recently reported (Paranjpe et al., 2013b), confirming a known high incidence of skeletal myopathy (Tsuchiya et al., 2005) and low incidence of thymic changes (Morton et al., 2004), and identifying additional vascular anomalies and mesenteric thrombosis as unique lesions in these mice. As BALB/cByJ mice are prone to cardiac dystrophic mineralization/calcification/calcinosis (Glass, 2013), a low incidence of this finding would also be expected, but has not been reported in this model.

Transformation Related Protein 53 Deletional (Trp53$^{+/-}$) Model (Formerly p53$^{+/-}$)

The Trp53 knockout mouse is commonly referred to as the p53 mutation, with both heterozygous ($^{+/-}$) and homozygous ($^{-/-}$) mutations available on multiple genetic backgrounds in mice, and several strains of rats. p53 protein is a transcriptional regulator of genes involved in cell cycle arrest, DNA repair, apoptosis, anti-angiogenesis, differentiation and gene stability. Mice completely lacking p53 protein develop a multitude of spontaneous tumors, particularly malignant lymphomas and sarcomas within 6 months-of-age and seldom live beyond 10 months. Heterozygous Trp53$^{+/-}$ mice have one wild type and one null allele, and partial protein production delays spontaneous tumor development until 9 months-of-age and extends the lifespan to 18-months (French et al., 2001). Validation of short-term carcinogenicity testing of 21 compounds by multiple routes was conducted using the partially congenic heterozygous B6.129-Trp53^{tm1Brd}N5, although this mutation is now available in N12 backcross fully congenic C57BL6 mice. In the presence of mutagenic carcinogens but not nonmutagenic carcinogens, tumors and hyperplastic lesions were induced in the Trp53$^{+/-}$ model after 26 weeks of continuous exposure (Storer et al., 2001). Due to foreign body carcinogenesis identified by the frequency of microchip transponder-induced aggressive subcutaneous sarcomas, Trp53$^{+/-}$ mice should not be used for carcinogenicity of medical devices or implants (Blanchard et al., 1999; Storer et al., 2001). Spontaneous tumor biology in Trp53$^{+/-}$ mice is well studied with genetic background and caloric restriction found to be substantive modifiers of tumor onset (Hursting et al., 2001) and incidence and type of tumors (Donehower et al., 1995; Mahler et al., 1998), and some tumors actually retain the wild type Trp53 allele (Venkatachalam et al., 2001). An understanding of the spontaneous pathology of mouse strains used in genetic engineering is important to interpretation of tumor and non-tumor findings in these alternative carcinogenicity assays. For the Trp53 models, this includes the C57BL6 and 129 substrains (Ward et al., 2000; Haines et al., 2001).

FVB/NTac-Tg(Hba-x-v-Ha-ras)TG.ACLed Transgenic Model (Tg.AC)

The Tg.AC model was created by transgenic insertion of a Harvey rat sarcoma viral onco-gene homologue (HRAS, formerly v-Ha-ras) with point mutations fused to a mouse zeta globulin promoter and an SV40 polyadenylation/splice sequence. This transgene bypasses the need for an initiation event and confers development of dermal papillomas after injury or dermal application of promoter carcinogens. Both the homozygous (Tg.AC$^{+/+}$) and hemizygous (Tg.AC$^{+/-}$) mice can be used to identify genotoxic and nongenotoxic carcinogens, but gene dose effect increases the incidence of tumors in homozygous animals. Although the incidence of external and internal spontaneous tumors is low, these mice develop a high incidence of rare tooth odontogenic tumors not present in wild type mice. Additionally, forestomach papillomas and epidermal squamous cell papillomas that occur at sites of fight wounds and chronic grooming in homozygous and hemizygous mice are also absent in wild type mice. Spontaneous lung adenocarcinomas and carcinomas occur in Tg.AC and wild type mice (Mahler et al., 1998; Tennant et al., 2001). Other tumors including lymphomas and erythroleukemia occur at a much lower incidence. The lack of certain tumors in the FVN/N wild type mice highlights the need to evaluate and understand the spontaneous pathology in the wild type of genetic modifications in mice used in short-term carcinogenicity assays (Mahler et al., 1996b; Ward et al., 2000). The Tg.AC model has also shown genotypic instability with appearance of a nonresponder genotype that has since been eliminated (Blanchard et al., 1998; Weaver et al., 1998; Tennant et al., 2001). In the ILSI/HESI initiative, 14 compounds were tested by topical and oral routes. Both mutagenic and nonmutagenic carcinogens were detected but this model did not detect rodent hepatocarcinogenicity with peroxisome proliferators. Regardless, this model is of particular usefulness for carcinogenicity testing of topical compounds.

Homozygous XPA Knockout (Xpa$^{-/-}$) Model

The XPA knockout mouse model on the C57BL6 background (C57Bl/6J-TgH(XPAIm)55Cmg) is based on a rare autosomal recessive human disease, xeroderma pigmentosum, complementation group A (XPA), in which DNA repair of UV-induced skin damage is absent or defective due to a mutation in the nucleotide excision repair (NER) gene (Steeg et al., 1998, 2001). The Xpa (homozygous Xpa$^{-/-}$ and heterozygous Xpa$^{+/-}$) and wild type mice develop a low incidence of internal lymphomas and adenomas. Xpa$^{-/-}$ mice exposed to UV light mimic cutaneous tumor susceptibility of XPA patients exposed to sunlight, with the development of basal and squamous cell tumors in patients and squamous cell papillomas and carcinomas in mice (Steeg et al., 1998). While the incidence of internal tumors is low in patients, Xpa$^{-/-}$ mice exposed to oral carcinogens reactive with the NER pathway develop lymphomas and adenomas, but require up to 9 months of exposure. Xpa$^{-/-}$ mice do not develop neurological problems reported in XPA patients and Xpa$^{-/-}$ mice develop liver and other adenomas not seen in XPA patients (Steeg et al., 1998). ILSI/HESI validated 13 compounds in mice by the oral route during 9 months of exposure with positive responses with the majority of immunosuppressive and hormonal human carcinogens but also one nongenotoxic animal carcinogen (putative human noncarcinogen). The Xpa$^{-/-}$ mice were also prone to overt toxicity with some of the carcinogens (van Kreijl et al., 2001). In order to increase the sensitivity of the Xpa$^{-/-}$ model, a double knockout mouse was created by the addition of the Trp53$^{+/-}$ mutation and considered to be a better model than the Xpa$^{-/-}$ model (Steeg et al., 2001).

XPA and Trp53 (Xpa$^{-/-}$/Trp53$^{+/-}$) Double Knockout Model

This model incorporates the lack of cell cycle control and defective apoptosis in the tumor suppressor Trp53$^{+/-}$ model with defective DNA repair mechanisms in the homozygous Xpa$^{-/-}$ model. These mice develop spontaneous tumors reflective of both defects including the Xpa-specific

lymphomas, bronchiolo-alveolar adenomas and adrenal cortical adenomas plus the Trp53-specific lymphomas and sarcomas (Steeg et al., 2001). Despite the synergistic effect on cancer susceptibility with this double knockout, only carcinogens restricted to these two pathways are likely to be detected. ILSI/HESI validated 10 compounds by the oral route during 9 months of exposure with positive responses with the genotoxic compounds. As with the Xpa model, the Xpa/Trp53 mice had positive responses with the majority of immunosuppressive and hormonal human carcinogens but were negative for the nongenotoxic animal carcinogen that was positive in the Xpa$^{-/-}$ mice. Overall, this double knockout was more sensitive than the Xpa$^{-/-}$ mouse model and the Xpa$^{-/-}$/Trp53$^{+/-}$ model was recommended for short term carcinogenicity testing, with the caveats of increased potential for toxicity to carcinogens and the need to extend exposure to 9 months (van Kreijl et al., 2001).

Other GMM Models of Carcinogenicity

Although not considered sensitive enough to be used for short-term carcinogenicity testing, Eμ-*pim*-1 transgenic C57BL/6 mice with insertion of an enhanced Pim-1 oncogene, may be suitable for carcinogenicity testing of lymphoid compartments (van Kreijl et al., 1998). Transgenic adenocarcinoma mouse prostate (TRAMP) mice (C57BL/6-Tg(TRAMP)8247Ng/J x FVB/NJ)F1/J express the simian virus 40 (SV40) large tumor T antigen (Tag) under the control of the rat probasin promoter, and phosphatase and tensin homolog (Pten) knockout mice are valuable models for prostate tumors (Shappell et al., 2004). Similarly, transgenic Erbb2 oncogene homolog 2 (HER-2/c-neu oncogene) and knock-in Brca1 and Brca2 mice have been used to model breast cancer (Blackshear, 2001). The adenomatous polyposis coli (Apc) gene inactivation model appears to be a model of colonic carcinoma when refined to limit tumor expression to the large intestine as was done in the Apc$^{580S/+}$ mouse (Xue et al., 2010). Finally, another model for carcinogenicity testing of chemicals that may be appropriate in limited circumstances is genetically modified zebrafish (Spitsbergen and Kent, 2003).

THERAPEUTIC PRODUCTS DERIVED FROM TRANSGENIC ANIMALS

While the predominant genetic manipulations for research and development have focused on rodents, transgenic animals have been used as bioreactors to produce biopharmaceuticals and vaccines (VanCott and Velander, 1998; Committee on Defining Science-based Concerns Associated with Products of Animal Biotechnology, 2002). Plasma-free recombinant antithrombin III produced in the milk of transgenic goats (*Capra hircus*) (ATryn®, rEVO Biologics) is the first transgenically derived therapeutic product to be approved in the United States and EU. Guidelines for the manufacturing and testing of therapeutic products derived from transgenic animals are similar to cell-based systems in that the genetic construct, founder lines, stability of the genetic insertion, and the safety, purity and potency of the final product need to be thoroughly described and documented (FDACBER, 1995; ICH, 1997, 1999a,b, 2011b; van Waterschoot and Schinkel, 2011)

GENETICALLY ANIMALS AS SOURCES OF FOOD, MEDICAL DEVICES AND XENOTRANSPLANTS

Concerns have been raised about GMA intended for use as food (meat, milk, eggs, or fish), potential disposition of excess or retired GMA created for biomedical production into the food stream, and in xenotransplantation of tissue products and organs. Potential issues include human contamination through chemical residues from animal drugs and pathogens (microorganisms used as insertional vectors or the unintentional introduction of microorganisms from the host), immunological perturbations (hypersensitivity and allergenicity) and environmental impact (Committee

on Defining Science-based Concerns Associated with Products of Animal Biotechnology, 2002). In food production, farmed genetically modified triploid (sterile) Atlantic salmon (*Salmo salar*) with rapid growth conferred by insertion of a growth hormone gene from Chinook salmon (*Oncorhynchus tshawytscha*; AquAdvantage® salmon), were recently deemed by the FDA to be a safe food source and without environment threats as per their guidance document (FDACVM, 2009). This acceptance remains controversially and the comment period for the environmental assessment on the New Animal Drug Application was extended an additional 60 days. As of this writing, the preliminary finding of no significant impact to the environment remains remains under review (FDA Animal & Veterinary, 2013). GMA used for medical devices and xenotransplantation require clear documentation of source qualifications, husbandry and records similar to GLP-based studies and testing for infectious agents. As with any cell products, stored or processed products require testing for identity, sterility, purity and potency and preclinical testing including tumorgenicity (FDACBER, 1995, 2003; ICH, 1997, 1999a,b, 2011b). Carcasses from genetically modified source animals are automatically excluded from the food stream due to the use of anesthesia for tissue and organ harvesting. Surplus animals or animals with failed genetic transfer would generally be considered medical waste, although the FDA Center for Veterinary Medicine might infrequently consider these animals for food use or rendering (FDACBER, 2003).

GENETICALLY MODIFIED RATS IN TOXICOLOGY PROGRAMS

Zinc-finger nuclease-induced gene targeting and homologous recombination in embryonic stem cells are techniques that have allowed transgenic and knockout rats to be reliably produced (Jacob et al., 2010). Specific models such as the inflammatory prone HLA-B27 transgenic rats could be used for efficacy testing and to model disease (O'Neill, 1997). Homozygous null (deficient) Trp53 rats (*SD-ITp53^{tmfsage}* and Crl:WI(UL)-*Tp53^{m1/Hubr}*) are available for in vivo research and carcinogenicity screening but validation and use of this model as a substitute for 2-year bioassays and the regulatory acceptance of this model are not yet available. Although the number of genetically modified models for discovery, ADME, pharmacology, drug-drug interactions and efficacy characterization in rats are currently limited compared to mice, the selection of available mutations in rats is expanding (Phenotypes & Models at RGD, 2013).

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Model Selection and Scaling (Allometry)

Shayne Cox Gad

CONTENTS

    This entire volume is directed at the premises that (1) animals can serve as accurate predictive models of toxicity in humans (or other species), (2) the selection of an appropriate species to use is key to accurate prediction in man, and (3) understanding the strengths and weaknesses of any particular model is essential to understanding the relevance of specific target organ toxicities to what would be expected in humans. Each of these premises requires some examination. Historic recognition of these facts (Priestly, 1772) has led to uses ranging from tasting food for poisons to the use of canaries in coal mines (Burrell, 1912).

It is a fundamental hypothesis of toxicology that adverse effects caused by chemical entities in animals are generally the same as those induced by those entities in humans. There are many who point to individual exceptions to this and conclude that the general principle is false. Yet, as our understanding of molecular biology advances and we learn more about the similarities of structure and function of higher organisms at the molecular level, the more it becomes clear that the mechanisms of chemical toxicity are largely identical in humans and animals. This increased understanding has caused some of the same people who question the general principle of predictive value to in turn suggest that our state of knowledge is such that mathematical models or simple cell culture systems could be used just as well as intact animals to predict toxicities in man. This last also misses the point that the final expressions of toxicity in humans or animals are frequently the summation of extensive and complex interactions on cellular and biochemical levels. Zbinden (1987) published extensively in this area, including a very advanced defense of the value of animal models. Lijinsky (1988) has reviewed the specific issues about the predictive value and importance of animals in carcinogenicity testing and research. Though it was once widely believed (and still is believed by many animal rights activists) that in vitro genotoxicity tests would replace animal bioassays for carcinogenicity, this is clearly not the case on either scientific or regulatory grounds, though our understanding of the underlying processes has advanced to the point that the use of a single species (the rat, rather than the rat and mouse) is rapidly become accepted. Though there are differences in the responses of various species (including humans) to carcinogens (Gregory, 1988), the overall predictive value of such results (when tempered by judgment) is clear. Additionally, though, many propose that there is little data to support that findings of toxicity in animals have been predictive of adverse effects in humans (because agents found toxic in animals are generally not knowingly given to humans). This is not the case (see Zbinden [1987] and Greaves et al. [2004], or refer to recent events surrounding azidothymidine, retinoids, or peroxisome proliferator-activated receptors [PPARs]). Upon close examination, most postmarketing "idiosyncratic" toxicities seen in humans were at least hinted at in earlier animal studies.

The principal problem in using animal models as predictors of toxicity in humans lies in the second major premise cited at the beginning of this chapter. As will be addressed in the first major section of this chapter, the theoretical basis for selection of the appropriate animal model is well established and often quoted (Plaa, 1976) but rarely adhered to. As the nine core chapters in this book should have established, each commonly used model species has both strengths and weaknesses. Probably, the greater weakness in the current practice of toxicity testing is a lack of care in selection of the appropriate model. Our third premise is this very point: each model species has strengths and weakness, and an understanding of these in evaluating findings in the species actually used is essential to establishing the relevance (or lack of relevance) of findings.

This is, of course, one reason that toxicity is generally evaluated in at least two species. As Plaa (1976) and Litchfield (1962) have pointed out, identical (or comparable) findings of adverse effects found in two separate and diverse model species have a much higher predictive power for the outcome of exposure in humans. A subset of this consideration (susceptibility factors) is addressed in the next chapter. Susceptibility factors are differences in either the animal model or human population (such as age, sex, disease, diet, physiological state, or strain) that can markedly influence the toxicity of a chemical entity and the course of an induced pathogenesis (Animal Clinical Chemistry Association, 1988). Many times the predictive value of our animal studies could be improved by altering elements of protocol or experimental design to allow for the existence of susceptibility factors in a target population. Another corollary of this third premise is that there are times when adverse effects have nothing to do with what will happen in humans. Clayson (1988) has reviewed the case for this in terms of carcinogenicity, but the argument is not limited to that.

MODEL SELECTION

The key points in selecting the most appropriate animal species as a model are to start by clearly identifying the objective of the studies to be performed and then to utilize all available information on both available model species and the mode of action and pharmacokinetics of the compound to be studied.

If the objective of a study is limited to acting for a screen or a "single end point" type assay (Gad and Chengelis, 1998), then a much wider range of species will be appropriate than if greater discrimination is required.

Given all that has been presented in the core chapters on test systems, the characteristics of different species and how one extrapolates from one species to another, the next obvious question is how are test species actually selected? An additional consideration that has grown in importance in recent years is the consideration of immunologic responses when the drug in question is a protein of other complex biologic (Satish et al., 2013).

The obvious theoretical best choice would be the species considered at risk, which would leave us with no difficulty in extrapolating from one species to another.* For some applications (veterinary agents or where the concern is for the effects of potential exposures to domesticated animals or wildlife), it is possible to take this approach. But for most cases, where the real-life concern is potential toxicity to humans, a laboratory animal species must be selected; as although drugs and chemicals are still occasionally administered de novo to people for experimental purposes, there are legal and ethical issues that make this a rare case indeed before at least some short-term toxicity data has been gathered in one or more species other than humans (National Academy of Sciences, 1975). Even then, initial toxicity tests carried out in humans are generally at low-dose levels compared to the intended potential therapeutic or other human exposure levels or the toxic doses predicted from animal experiments. The design of acute toxicity studies in which humans are used is commonly directed at the evaluation of alterations in hematology and blood chemistry, measurable physiological variables, and the analysis of the agent and its metabolites in the blood, urine, feces, and tissues (Nosal and Hladka, 1968; Rider et al., 1969). With pesticides and a few other environmental agents, useful human acute toxicity data has been obtained by the study of accidentally exposed individuals (Brown, 1980).

What then would constitute the best choice of models? There is a set of characteristics that most would agree constitute the "ideal" animals on scientific grounds. These include (1) similarity of absorption, distribution, metabolism, and excretion (ADME) to humans; (2) sensitivity of the species to the agent closely resembling that of humans (especially if the drug is operative at receptor present only in humans and a few other species); (3) evolutionary level of the animal; (4) ability of the species to express the full range of responses that humans would (such as emesis); (5) ability to make all pertinent measurements in a meaningful way; and (6) stages of the life span should correlate directly to those of humans. In general, larger species (dog, pig, and primate) are more often better predictors than rodents. And interestingly, dogs are better predictors than primates for across a range of compounds (Greaves et al., 2004).

However, there is also a set of desired characteristics for an ideal species to possess from a technical management point of view. These criteria are as follows:

1. Have a low body weight. The weight of the experimental animal is so important because during the early stages of development of new commercial chemicals only small quantities of the test material may be available.
2. Be easy to bleed and large enough to supply a sufficient amount of blood for analysis on a periodic basis (though recent improvements in sampling and analytical methods have made this an easier issue to manage).

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* Though, as will be seen later in this chapter under "Limitations of Models," not all members of the same species (even disregarding sex and strain differences) respond the same.

3. Be easy to obtain or breed a homogenous supply of healthy animals and maintain them in the laboratory.
4. Be easy to handle and to administer test agents to by the various desired routes.
5. Should have a short life span.
6. Physiology and metabolism should approximate those of humans.
7. Should not pose a disease threat to handlers.

Each of these ideal features is secondary to the desire to have a model that responds exactly as our target species. However, there is no animal species that mimics humans in all respects, so that the ultimate choice depends on the balance of conflicting factors. For example, consider the case of the selecting a model to predict the effects of agents on the gastrointestinal (GI) tract of humans (Fara et al., 1988). There is no single best model, with the common species (rat, mouse, and dog) generally being more sensitive than humans to such agents as nonsteroid anti-inflammatory drugs (NSAIDs). It is all too easy to suggest that the animal of choice should fit the criteria enumerated earlier, but in actuality, these are empty words. Actual selections are made generally on practical and "political" criteria rather than these logical points.

After a scaled human dose (such as the FDA's human equivalent dose [HED]) has been determined from the no observed adverse effect levels (NOAELs) from all toxicology studies relevant to human potential risks, the next step is to pick a single HED for subsequent risk assessment. This HED should be chosen from the most appropriate species. In the absence of data on species relevance, a default position is that the most appropriate species for deriving the potential human exposure.

Human potential risk in adult healthy volunteers is the most sensitive species (i.e., the species in which the lowest HED can be identified).

Factors that could influence the choice of the most appropriate species rather than the default to the most sensitive species include (1) differences in the ADME of the therapeutic between the species; (2) class experience that may indicate a particular model is predictive of human toxicity; or (3) limited biological cross-species pharmacologic reactivity of the therapeutic. This latter point is especially important for biological therapeutics as many are human proteins that bind to human or nonhuman primate targets (see ICH guidance S6).

When determining the potential human risks in the absence of actual human data, absorption, distribution, and elimination parameters will not be known for humans. Comparative metabolism data, however, might be available based on in vitro studies. These data are particularly relevant when there are marked differences in both the in vivo metabolite profiles and HEDs in animals. Class experience implies that previous studies have demonstrated that a particular animal model is more appropriate for the assessment of safety for a particular class of therapeutics. For example, in the nonclinical safety assessment of the phosphorothioate antisense drugs, the monkey is considered the most appropriate species because monkeys experience the same dose-limiting toxicity as humans (i.e., complement activation), whereas rodents do not. For this class of drugs, the potential safe exposure to man would usually be based on the HED for the NOAEL in monkeys regardless of whether it was lower than that in rodents, unless unique dose-limiting toxicities were observed with the new antisense compound in the rodent species. Similarities of biochemistry and physiology between the species and humans that are relevant to the limiting toxicities of the therapeutic should also be considered under class experience. If a species is the most sensitive but has differences in physiology compared to human that sensitizes it to the therapeutic, it may not be the most appropriate species for selecting the potential safe exposure to man.

The data necessary to make decisions based on practical considerations has largely been incorporated into the tables in the core chapters of this book. Economic considerations turn out to be among the most important. These include the cost of the animal and its upkeep, availability of test animals, housing requirements, and a host of other factors that tend to push selection toward smaller, established test animal species.

Table 16.1 Approximate Distribution of the Vertebrate Animal Species

Category	No. of Distinct Species
Fish	23,000
Amphibians	2,000
Reptiles	8,500
Mammals	4,500
All vertebrates	43,000

Source: Rothschild, L., *A Classification of Living Animals*, Longmans, London, U.K., 1961.

The possibilities for selection are, of course, much wider. The subkingdom of vertebrate animals alone contains a great number of species, which can be classified into distinct categories, as shown in Table 16.1.

But only a few of this multitude of possible species has actually been employed at any time. And the eight species that are discussed in depth in this chapter represent virtually all (99.9%) of the animals currently used in toxicology. Why is this?

How Species Are Actually Selected

There are two major sets of factors that actually drive the process of model selection in acute toxicology, with rare exceptions.

First, economic considerations such as ease of commercial production and availability, housing, life span, etc. have, as was pointed out earlier, favored the use of small laboratory animals. In the resulting enthusiasm for establishing rodents as the satisfactory test models, toxicologists have conveniently overlooked the fact that there have been few studies correlating the toxicity of specific compounds in humans and these animal species. The available information suggests a moderate-to-fair direct correlation (Litchfield, 1961, 1962). Difficulties in validating alternative or new test systems have tended to preclude any improvement of the model systems that are employed. There is an urgent need for a nonrodent species with a life span of up to 5 years that does not have the problems inherent in the dog or primate. Ferrets, marmosets, miniature pigs, and a number of other species have been investigated during the last 20 years, with incorporation into more common use (for the minipig and ferret). There clearly remains an opportunity for the commercial animal breeder here, but it may be that the essential criteria are impossible to meet and the underlying societal inertia is too great. Stevenson (1979) has discussed these aspects in the wider context of general toxicology.

The second major set of factors can only be classified generally as custom or habit. What the scientists and technicians are used to using, and what the regulators are used to interpreting data from, is generally what we tend to continue doing. The resulting inertia is the greatest impediment not only to proper model selection, but also to adaptation of new or improved study designs and to the development and use of in vitro models. The frequently raised issue of "validation" for any proposed change in what the science of toxicology does is all too often more accurately stated as, "Show us that it gives us the same answers—we know how to deal with those, even if they are wrong."

To fulfill these two sets of factors, animals are then actually selected for acute testing based on the following steps:

1. Which species will meet test design needs?
2. What is species availability?
3. How much test compound is available? If the amount is limited, the smallest (body weight) species that will meet other needs will most often be selected. However, there are some cases where less compound may be required to achieve desired levels in larger species than in smaller species (due to pharmacokinetic).

4. What species is the least expensive, both in terms of costs directly associated with the animals and indirect costs (the easier an animal is to handle, e.g., the lower are the labor costs associated with the study). There are some special cases of species selection being based on expense. The most common of these is where the compound (usually a drug) being studied is very expensive or in extremely short supply. As a result, using the smallest possible animal reduces compound use. In this sense, small primates are often "less" expensive than dogs.

5. Will the species selected meet regulatory guidelines (usually easy, as these either dictate a species for a particular test or simply specify rodent or nonrodent) and have regulatory "acceptance" (not so easy)?

6. What have we used in the past? This question usually actually comes first, generating a list of candidate species.

7. Under new emphasis on not using animals unnecessarily (Animal Welfare Act, 1987 as amended) more care is being taken in species selection.

Special Cases in Species Selection

There are a number of the routes of exposure that have a particular species that is by habit (frequently based on a form of folklore) especially favored.

In inhalation, there are several special considerations of anatomy and physiology that dictate model selection. The following three factors should be strongly considered in species selection for inhalation studies:

1. Mouth (10 µ filtration) versus nose (3 µ filtration) breathers. A human versus rodent comparison, with considerable resulting differences in both particular/droplet filtration and regional absorption.

2. Number of "daughter" generations of air passages. These are the number of successive times that air passages in the respiratory tree branches. There are 35 in the human.

3. Distribution of major compartments in the respiratory tract. Humans have
   a. Nasopharyngeal
      i. Nasal cavity
      ii. Mouth
   b. Tracheobronchial
      i. Larynx
      ii. Trachea
      iii. Bronchials
   c. Alveolar sacs
      i Pulmonary

The rat is far and away the most common species used in inhalation, even though it is an obligatory nose breather and its respiratory morphophysiology is much different than that of humans (it has five lung lobes and a total lung surface area of 7.5 m^2—10% of that of humans).

Folklore says that primates are the best inhalation model for humans. However, the closest similarity in respiratory structure and function is probably found in the horse or donkey. Besides the rat, commonly used species include none of these, however. Rather, the mouse and dog are the only other commonly used species in inhalation.

Likewise, dermal studies are by custom performed on the rabbit because its dermal absorption is "greater" than that of humans, making it the most sensitive model. As has been presented in a review by Gad and Chengelis (1998), this in fact is not the case.

Neither is it true that in the general case the dermal absorption and skin morphology of the pig most resemble humans (as originally reported by Bartek et al., 1972), though the minipig is far and away the preferred model for dermal studies (McAnulty et al., 2013). Rather, the answers as to

which species is either most sensitive or most resembles humans depend very much on the structural characteristics of compounds in question. Recent efforts and suggestions by some that all toxicity tests be performed on a common species, such as the rat, merit wider consideration. If we as scientists are not willing or able to select models on a scientific basis of what will provide us with the best prediction of what would happen in humans, then the argument of at least using a single common model that we understand the weaknesses of becomes a compelling one.

Caution

Having considered the general process of model selection, one should next be aware of the limitations and peculiarities of the common models and of some of the variations that occur within a species due to differences between strains of animals.

LIMITATIONS OF MODELS

Despite our best efforts, when human exposures to a chemical entity (such as a drug) occur, the results do not always come near to what was expected based on animal studies. For the population as a whole, there are a number of possible explanations. Some of these are presented in Table 16.2.

An example of these types of problems in the extrapolation of toxicity data from one species to another can be found in published studies on fenclozic acid, which was a potential new anti-inflammatory drug (Alcock, 1971). No adverse effects were observed to occur in the mouse, rat, dog, rhesus monkey, patas monkey, rabbit, guinea pig, ferret, cat, pig, cow, or horse, but the drug caused acute cholestatic jaundice in humans.

Beyond the difficulties in extrapolating from one or more test animals populations to the overall human population, there are a number of limitations to the standard model populations that are imposed by two forms of "good scientific practice" that is employed in conducting toxicity studies. Both of these practices have as their rationale the maximization of the sensitivity of the test system, with the underlying good intention of therefore providing the greatest possible protection to people. This is not actually the effect, however.

Table 16.2   Some Reasons Why Data Obtained in Animal Studies Does Not Always Match Human Experiences

1. The animal species selected differ in response from humans. The same measurement or experiments in a different animal species may have been more predictive.
2. Differences in absorption, distribution, and/or metabolism may be present.
3. The anatomy involved in the model may differ from that in people.
4. Different animal strains of the same species may generate different results.
5. The pathological nature of any lesions produced may differ at either a macroscopic or microscopic level.
6. There may be critical differences between the species at subcellular, cellular, receptor, or physiological levels that lead to different responses. This is particularly true in terms of our current use of clinical chemistry findings to identify "target organs" in animals when these enzymes may not have the same relationship to pathogenesis in animals as in humans. Consider, for example, the animal clinical chemistry associations' review of hepatic function and damage indicators in animals species "correct."
7. Experimental conditions in the animal model may yield qualitatively different data over the course of several experiments, and it may be unclear which set is.
8. The "dose" required to produce the observed results in animals is never achieved in humans.
9. The target dose in humans cannot be achieved in test animals.
10. The human population we are concerned about may differ from the population in general, and in so doing may have special characteristics, which were not adequately represented in our animal model population.

Table 16.3   Reasons Why High-Dose Toxicity Testing Is Usually Not Predictive of Human Effects

1. Solubility of the compound may be limiting.
2. Kinetics may be nonlinear (e.g., an enzyme may be saturated), and absorption may be decreased.
3. Michaelis–Menten kinetics may be applicable, and the blood levels may be greater than predicted in animals.
4. Metabolites formed in the animal studies may cause toxicity that would not occur with lower doses (high doses of phenacetin are one of many examples).
5. Detoxification mechanisms in the liver or elsewhere may be depleted or saturated (examples are high doses of acetaminophen in the liver or of hexavalent chromium in the lungs).
6. Bioavailability of the dose form may be entirely different at lower doses due to local physiological effects (such as irritation) in the high-dose animal studies.
7. High-dose levels in animals may overwhelm organ systems, which would not be affected at lower doses, causing effects that serve to make those seen at lower blood levels.

The first of these practices is that toxicity testing has traditionally been performed at high doses. Even in acute toxicity studies that have the objective of predicting potential target organs and mechanisms of toxicity for humans at much lower doses, the study is considered suspect if all (or for some people, any) animals survive at the highest dose level tested. This use of a maximum administrable dose and large fractions thereof is a spillover from carcinogenesis testing and times when our ability to detect effects was crude. It can frequently produce errors or difficulties in prediction of effects in people, such as those in Table 16.3.

The second practice is that of using test animal populations that are as homogeneous as possible. This current strategy for quantifying toxicity for the most part evaluates toxicity in homogeneous populations, whether in animals or in humans, whether in vitro or in vivo. Such an approach minimizes the expression of background biological variability and, therefore, generates the most readily quantifiable and "sensitive" estimates of predicted toxicity. In actual target populations that are more heterogeneous than the model population with respect to, among other things, susceptibility and resistance to the compound in question, this may not be the case. The rationale may not be truly applicable to effects on humans, where toxicity in even a relatively small susceptible population would not be acceptable. Adjustments for this wider range of susceptibility in the population of potentially exposed humans is most commonly accounted for as being part of what is involved in the use of safety factors in setting allowable limits for human exposure. This may not be either accurate or adequate.

The underlying view is that a potentially toxic exposure occurs with the interaction of the chemical and a model population in a particular space and time. This experimental event of exposure must be characterized by the range of "dose"; type of exposure; characteristics of the exposed population (weight, sex, strain, etc.); the biological characteristics of the effect at the molecular, cellular, tissue, organ, individual, and population levels; and over a spectrum of effects from physiological through pathological and behavioral. The toxicity of the exposure must be characterized in terms of the severity of the effect—for example, clinical signs, disability, and/or death. Later, someone must consider the relevance and acceptability of an observed effect to human society. As a corollary of the principles of experimental design, each variable in a protocol is rigidly fixed within a narrow range. And we use a "high-class" test population—the healthiest, most nutritionally adequately fed young adult animal population possible. These laboratory animals have been carefully bred to make them as genetically defined as possible and are maintained in clean cages under narrowly controlled environmental conditions. Thus, toxicologists traditionally utilized a very robust and (at best) narrowly representative population of animals under the best of environmental conditions.

How then do we predict for the real-life susceptibility of human populations that we are most concerned about, or at least allow for them in our predictions? In many cases, perhaps one should utilize special "at-risk" model populations in such tests. For example, if older individuals or those

with compromised cardiovascular function are known to constitute a significant part of the potentially exposed human population, then study designs should incorporate groups of animals that can serve to determine if such conditions render the animals (and therefore potentially people) more susceptible to toxic effects or if it changes the nature of the expression of the toxic effect.

Susceptibility to an effect at any particular moment in a biological organism is determined by three sets of variables affecting the biological state of the organism at the time of exposure that are largely "invisible" to the outside world: genetic constitution, previous life experience, and momentary physiological state. Genetic constitution is determined by factors of strain, species, family, congenital abnormalities, and any acquired alterations. Species factors are the result of the selection of major genetic components over the course of evolution and have already been discussed. Strain factors have largely been determined by selective breeding for concentration of genetic characteristics by the laboratory animal breeder, and considerations of strain will be presented later in this chapter.

But there are a number of factors that are part of the other two aforementioned variables (life experience and momentary physiological state) that are not generally characteristically represented or considered in our test animal population and yet do exist in humans and do contribute to the biological outcome of a chemical exposure. These can be considered "susceptibility factors." Chapter 14 will explore the problem of susceptibility in detail.

CROSS-SPECIES EXTRAPOLATION

For all the other words in this volume about the relevance of the test systems that are described, one should never forget that none of the animal species we use is other than a model for the human being. And ultimately, the continued use of animals in predictive testing must depend on how well we can use the data from these animal models to predict the outcome in people. The activity of transforming results in members of one animal species (say, rats) to one or more populations of another species (such as people) is called cross-species extrapolation or scaling (though technically scaling is actually limited to the act of making adjustments for differences in sizes or rates). There are multiple ways of doing these (Garattini, 1985; Hottendorf, 1987; Mitruka et al., 1976; Rozman, 1988; Smith, 1974; Sharma and McNeill, 2009; Schneider et al., 2004; Schrag and Regal, 2013).

Each step in the scaling process adds an additional degree of uncertainty to the final product. Wise and prudent scientific practice calls for at least three courses of action in seeking to give the best quality (i.e., least uncertain) final product in the form of what does this mean to humans. These courses of action are as follows: (1) have as few steps in the prediction process as possible, (2) have as little uncertainty as possible associated with each step, and (3) understand the places and ways in which the selected model fails as a predictor. Each of these courses of action is not only an integral part of the extrapolation process, but also contributes heavily to proper model (test species) selection.

All the efforts so far in this book have focused primarily on the aforementioned Step 2 and part of Step 3—performing various tests in a manner that gives us the least imprecise and most relevant data possible, and understanding the associated weaknesses of the model systems we employ. As such, our efforts and resulting extrapolations to this point have generally been for the animal species that data were being developed in. Ultimately, it is necessary to predict what these data would mean in humans. With the wide range of effects we are concerned about here, what conversion factor (or factors) can we derive that would allow us to equate "x" dose or exposure in rats or "y" effect in dogs with what would be seen in humans at the same or different doses? The tools at hand for the effort consist of a collection of mathematical methods (generally based on either body weight or body surface area as a means of quantitatively bridging the gap) and a set of logical and empirical rules that have been developed over the years.

The mathematical aspects will be addressed primarily in this section of the chapter, whereas the "rules" will appear in the sections that follow. Though there is some scientific basis for these

mathematical conversions, it is not on a point-for-point basis and from one to two orders of magnitude of uncertainty are generally involved. Such extrapolations clearly have both quantitative and qualitative aspects, and the rules seek to limit the uncertainty about the qualitative aspects. Some form of pharmacokinetic and metabolic study would provide us with active agent concentrations at target organ sites as well as other information of value in safety assessments (Hawkins and Chasseaud, 1985). While this would be the best approach to such qualitative modeling, even these methods have both difficulties and limitations of their own accompanying their clear advantages (Gillette, 1979) and are expensive and generally not available for support of most cases of data interpretation. An extension of this approach is that of physiological modeling (Gibson and Starr, 1988), which seeks to develop a quantitative compartmental model for each identified organ or organ system.

The qualitative aspects of species-to-species extrapolations are best addressed by a form of classification analysis tailored to the exact problem at hand. This approach identifies the known physiological, metabolic, and other factors that may be involved in the risk-producing process in the model species (e.g., the skin sensitization process in the guinea pig), establishes the similarities and differences between these factors and those in humans, and comes up with means to bridge the gaps between these two (or at least identifies the fact that there is no possible bridge).

Table 16.4 presents an overview of the classes of factors that should be considered in the initial step of a cross-species extrapolation. Examples of such actual differences that can be classified as one of these factors are almost endless.

Table 16.4   Classes of Factors to Be Considered in Species-to-Species Extrapolations of Toxicity

I. Relative sensitivity of model (compared to humans)
    A. Pharmacological
    B. Receptor
    C. Life span
    D. Size
    E. Metabolic function
    F. Physiological
    G. Anatomical
    H. Nutritional requirements
    I. Reproductive and developmental processes
    J. Diet
    K. Critical reflex and behavioral responses (such as emetic reflex)
    L. Behavioral
    M. Rate of cell division
    N. Other systemic defense mechanisms
    O. Can end point of interest be expressed in both species?
II. Relative population differences
    A. Size
    B. Heterogeneity
    C. Selected nature of test population (model populations are "high class" compared to the human population)
III. Differences between test and real-world environment
    A. Physical (temperature, humidity, etc.)
    B. Chemical
    C. Nutritional

The absorption of compound from the GI tract and from the lungs is generally comparable among vertebrate and mammalian species. There are, however, differences between herbivorous animals and omnivorous animals due to the differences in stomach structure. The problem of distribution within the body probably relates less to species than to size and will be discussed a little later. Primarily, endogenous metabolism, xenobiotic metabolism of foreign compounds, metabolic activation, or toxification/detoxification mechanisms (by whatever name) are perhaps the critical factors, and this can differ widely from species to species. The increasing realization that the original-administered compound is not necessarily the ultimate toxicant makes the further study of these metabolic patterns critical.

In terms of excretory rates, the differences between the species are not great; small animals tend to excrete compounds more rapidly than large animals in a rather systematic manner. Boxenbaum (1982) has generalized this to the concept of "pharmacokinetic time," which is related to relative body size. Small molecules are generally distributed and cleared through the circulatory system, and allometric scaling of the volume of distribution across species is generally a successful method for scaling, though regulatory acceptance of such calculations is limited (Schrag and Regal, 2013). Large molecules tend to be more commonly distributed by the lymphatic system and present a different set of problems.

The various cellular and intracellular barriers seem to be surprisingly constant throughout the vertebrate phylum. In addition, it is becoming increasingly clear that the various receptors, such as DNA and the neurotransmitters, are comparable throughout the mammalian species.

There are life-span (or temporal) differences that are not considered adequately, nor have they been in the past. It takes time to develop, for example, a cellular immune response and at least some of this time may be taken up by actual cell division processes. Cell division rates appear to be significantly higher in smaller animals. Mouse and rat cells turn over appreciably faster than human cells do—perhaps at twice the rate. On the other hand, the latent period for expression of many effects is also much shorter in small animals than in large ones.

Another difficulty is that the life span of humans is from 4.4 to 66.0 times (Gad and Chengelis, 1998) that of common test species. Thus, there is generally a much longer time available for many toxicities to be expressed or developed in people than in test animals. These sorts of temporal considerations are of considerable importance, and this area of chronotoxicology has not yet begun to really be explored.

Body size, irrespective of species, seems to be important in the rate of distribution of foreign compounds throughout the body. A simple example of this is that the cardiac output of the mouse is on the order 1 mL/min, and the mouse has a blood volume of about 1 mL. The mouse is, therefore, turning its blood volume over every minute. In humans, the cardiac output per minute is only 1/20th of the blood volume. So, the mouse turns its blood over 20 times faster than the human, which has clear implications for the comparative rates at which xenobiotics are systemically distributed in these two species.

Another aspect of the size difference which should be considered is that the large animal has a much greater number of susceptible cells that may interact with potential toxic agents, though there are also a proportionately increased number of "dummy" (hyporesponding) cells.

Roll (1979), Oser (1981), and Borzelleca (1984) have published articles reviewing such factors, and Calabrese (1983) had published an excellent book on the subject.

Having delineated and quantified species differences (even if only having factored in comparative body weights or relative body surface areas), one can now proceed to some form of quantitative extrapolation (or scaling).

Historically, there have been two major approaches to scaling for use with general toxicities. These are by body weight and by body surface area (Calabrese, 1983; Schmidt-Nielsen, 1984). Both of these

are single variables or 2D models, and represent alternate simple forms of what are called allometric equations. Davidson et al. (1986) have presented the generalized form of such equations as

$$Y = aW^n$$

where
    W is the body weight
    n is the slope of the derived line
    a is a scaling factor

Certain authors (e.g., Yates and Kugler, 1986) proposed that a multidimensional model would be more accurate. Such a form of allometric equation is probably too complicated for use in most cases in toxicology, however, and its use would be inappropriate considering the relatively imprecise nature of the data generated.

The body weight approach has historically been the most common general approach to scaling in toxicology—particularly in regulatory toxicology testing. There are several ways to perform a scaling operation on a body weight basis; the most often employed being to simply calculate a conversion factor (K) as

$$K = \frac{\text{Weight of human} \left(70 \text{ kg "standard"}\right)}{\text{Weight of average test animal}}$$

More exotic methods for doing this, such as that based on a form of linear regression, are reviewed by Calabrese (1983), who believes that the body weight method is preferable, Sharma and McNeill (2009), Schneider et al. (2004), and Schrag and Regal (2013).

A difficulty with this approach is that the body weights of any population of animals or people change throughout life, and in fact even at a common age will present considerable variation. Custom is, therefore, to use an "ideal person" (70 kg for men and 50 kg for women now set as a "standard" of 60 kg) or "ideal" animal weight (for which there is considerable less consensus).

The alternative is the body surface area methods, which attempt to factor in differences in metabolic rates based on the principle that these changes are in proportion to body surface area (since as the ratio of body surface area to body weight increases, relatively more energy is required to maintain a constant body temperature). As long ago as 1938, Benedict published a comparison of body weight versus basal metabolic rates for species from mice to elephants that showed a linear relationship between the two variables. Pinkell (1958) and Freireich et al. (1966) later found a similar relationship for effective/tolerated doses of cancer chemotherapeutics, and most recently Otterness and Gans (1988) have reported that the effective dose of NSAIDs could be scaled between species and models by surface area. There are several methods for making such conversions, each having a ratio of dose to the animal's body weight (in mg/kg) as a starting point, resulting in a conversion factor with Mg/M^2 as the units for the product of the calculations.

The Environmental Protection Agency version of the surface area scaling equation has generally been calculated as

$$(M_{human}/M_{animal})^{1/3} = \text{Surface area}$$

where M = mass in kilograms. Another form is calculated based on constants that have been developed for a multitude of species of animals by actual surface area measurements (Spector, 1956). The resulting formula for this approach is

$$A = KW^{2/3}$$

where

A is the surface area in square centimeters

K is a constant, specific for each species

W is the body weight in grams

A scaling factor is then simply calculated as a ratio of the surface area of a human over that of the model species.

Direct measurements of indicators of damage (much as tissue DNA damage; Visek, 1988 and Slaga, 1988) have also been proposed as the ultimate means of a cross-species scaling of exposures.

In late 2002, the U.S. FDA (FDA, 2002) released a draft guideline for cross-species extrapolation in using animal safety data to set initial closer for clinical trials in man, called the HED (or human equivalent dose) approach. This was also heavily based on the work of Boxenbaun and DiLea (1995) and Mahmood (2004). After the NOAELs in the relevant animal studies have been determined, they are converted to HEDs. A decision should be made regarding the most appropriate method for extrapolating the animal dose to the equivalent human dose. Toxic end points for therapeutics administered systemically to animals, such as the maximum-tolerated dose (MTD) or NOAEL, are usually assumed to scale well between species when doses are normalized to body surface (i.e., mg/m^2). The basis for this assumption lies primarily with the work of Freireich et al. (1966), Schein et al. (1970) and Belehradek (1957). These investigators reported that, for antineoplastic drugs, doses lethal to 10% of rodents (LD_{10}) and MTDs in nonrodents both correlated with the human MTD when the doses were normalized to the same administration schedule and expressed as mg/m^2. Despite the subsequent analyses showing that the MTDs for this set of drugs scale best between species when doses are normalized to $W^{0.75}$ rather than $W^{0.67}$ (inherent in body surface area normalization), normalization to body surface area has remained a widespread practice for estimating an HED based on an animal dose.

An analysis of the impact of the allometric exponent on the conversion of an animal dose to the HED was conducted. Based on this analysis and on the fact that correcting for body surface area increases clinical trial safety by resulting in a more conservative starting dose estimate, it was concluded that the approach of converting NOAEL doses to an HED based on body surface area correction factors (i.e., $W^{0.67}$) should be maintained for selecting starting doses for initial studies in adult healthy volunteers. Nonetheless, use of a different dose normalization approach, such as directly equating the human dose to the NOAEL in mg/kg, may be appropriate in some circumstances. Deviations from the surface area approach should be justified. The basis for justifying direct mg/kg conversion and examples in which other normalization methods are appropriate is described in the following subsection.

Although normalization to body surface area is an appropriate method for extrapolating doses between species, consistent factors for converting doses from mg/kg to mg/m^2 have not always been used. Given that body surface area normalization provides a reasonable approach for estimating HED, the factors used for converting doses from each species should be standardized. Since surface area varies with $W^{0.67}$, the conversion factors are therefore dependent on the weight of the animals in the studies. However, analyses conducted to address the effect of body weight on the actual body surface area–conversion factor demonstrated that a standard factor provides a reasonable estimate of the HED over a broad range of human and animal weights. The conversion factors and divisors shown in Table 16.5 are therefore recommended as the standard values to be used for interspecies dose conversions for NOAELs in Center for Drug Evaluation and Research and Center for Biologics Evaluation and Research. These factors may also be applied when comparing safety margins for other toxicity end points (e.g., reproductive toxicity and carcinogenicity) when other data for comparison (i.e., area under the curves [AUCs]) are unavailable or are otherwise inappropriate for comparison.

Table 16.5   Conversion of Animal Doses to HEDs Based on Body Surface Area

Species	To Convert Animal Dose in mg/kg to Dose in mg/m², Multiply by km below	To Convert Animal Dose in mg/kg to HED[a] in mg/kg, either	
		Divide Animal Dose by	Multiply Animal Dose by
Human	37	—	—
Child (20 kg)[b]	25	—	—
Mouse	3	12.3	0.08
Hamster	5	7.4	0.13
Rat	6	6.2	0.16
Ferret	7	5.3	0.19
Guinea pig	8	4.6	0.22
Rabbit	12	3.1	0.32
Dog	20	1.8	0.54
Primates:			
Monkeys[c]	12	3.1	0.32
Marmoset	6	6.2	0.16
Squirrel monkey	7	5.3	0.19
Baboon	20	1.8	0.54
Micropig	27	1.4	0.73
Minipig	35	1.1	0.95

[a] Assumes 60 kg human. For species not listed or for weights outside of standard ranges, HED can be calculated from the formula:

[b] This km is provided for reference only since healthy children will rarely be volunteers for Phase 1 trials.

[c] For example, cynomolgus, rhesus, stumptail, etc.

HED = animal dose in mg/kg × (animal weight in kg/human weight in kg)$^{0.33}$.

The factors in Table 12.5 for scaling animal NOAEL to HEDs are based on the assumption that doses scale 1:1 between species when normalized to body surface area. However, there are occasions for which scaling based on body weight (i.e., setting the HED [mg/kg] = NOAEL [mg/kg]) may be more appropriate. To consider mg/kg scaling for a therapeutic, the available data should show that the NOAEL occurs at a similar mg/kg dose across species. The following factors should be satisfied before extrapolating to the HED on an mg/kg basis rather than using the mg/m² approach. Note that mg/kg scaling will give a 12-, 6-, and 2-fold higher HED than the default mg/m² approach for mice, rats, and dogs, respectively.

NOAELs occur at a similar mg/kg dose across test species (for the studies with a given dosing regimen relevant to the proposed initial clinical trial).

If only two NOAELs from toxicology studies in separate species are available, then one of the following criteria should also be true:

- The therapeutic is administered orally and the dose is limited by local toxicities. Gastrointestinal (GI) compartment weight scales by $W^{0.94}$. GI volume determines the concentration of the therapeutic in the GI tract. It is thus reasonable that the toxicity of the therapeutic would scale by mg/kg ($W^{1.0}$).
- The toxicity in human (for a particular class) is dependent on an exposure parameter that is highly correlated across species with dose on an mg/kg basis. For example, complement activation by systemically administered antisense oligonucleotides in human is believed to be dependent upon Cmax (Geary et al., 1997). For some antisense drugs, the Cmax correlates across nonclinical species with mg/kg dose an in such instances mg/kg scaling would be justified.
- Other pharmacologic and toxicologic endpoints also scale between species by mg/kg for the therapeutic. Examples of such endpoints include the MTD, lowest lethal dose, and the pharmacologically active dose.

Other Exceptions to mg/m² Scaling between Species

Therapeutics administered by alternative routes (e.g., topical, intranasal, subcutaneous, intramuscular) for which the dose is limited by local toxicities present different problems. Such therapeutics should be normalized to concentration (mg/area of application, for instance) or amount of drug (mg) at the application site. Therapeutics administered into anatomical compartments have little subsequent distribution outside of the compartment. Examples are intrathecal, intravesical, intraocular, intrapleural, and intraperitoneal administration. Such therapeutics should be normalized between species according to the compartmental volumes and concentrations of the therapeutic. Biological products administered intravascularly with M_r > 100,000 daltons. Such therapeutics should be normalized to mg/kg.

Body Weight

Accurate conversion of mg/kg dose to mg/m² dose depends on the actual weight (and surface area) of the test species. A popular formula for converting doses is

$$\text{(i) mg/m}^2 = \text{km} \times \text{mg/kg}$$

where km = 100/K × W0.33, where K is a value unique to each species.

Or km = 9.09 × W0.365, where a K value unique to each species is not needed.

The km is not truly constant for any species but increases within a species as body weight increases. The increase is not linear but increases approximately proportional to $W^{2/3}$. For example, the km in rats varies from 5.2 for a 100 g rat to 7.0 for a 250 g rat. Strictly speaking, the km value of 6 applies only to rats at the "reference weight" of 150 g. For standardization and practical purposes, a fixed km factor for each species is preferred. An analysis was undertaken to determine the effect of different body weights within a species on the conversion of an animal dose to the HED using km factors. The km factor was calculated for a range of body weights using km = 100/K×W0.33. In Table 13.6, a working weight range is shown next to the reference body weight. This is the range within which the HED calculated by using the standard km value will not vary more than ±20% from that which would be calculated using a km based on exact animal weight. This is a relatively small variance considering dose separation generally used in deriving the NOAEL, in toxicology studies, which are often twofold separations. For example, suppose a NOAEL in rats is 75 mg/kg and the average rat weight is 250 g. The km for a 250 g rat is 7.0.

$$\text{HED} = 75 \times (7/37) = 14 \text{ mg/kg in humans.}$$

Using the standard km of 6 for rats,

$$\text{HED} = 75 \times (6/37) = 12 \text{ mg/kg in humans.}$$

The HED calculated with the standard km of 6 is within 15% of the value calculated using the actual km of 7. The body weights producing km factors for which the nominal, integer conversion factor was within 20% of the calculated factor covered a broad range (Table 13.6). This working weight range encompassed the animal weights expected for the majority of studies used to support starting doses in humans.

For the typical species used in nonclinical safety studies, Table 13.6 also shows the body surface area in m² for an animal at a particular "reference" weight. For example, a 400 g guinea pig has a body surface area of approximately 0.05 m². These values come from published sources with

Table 16.6   Conversion of Animal Doses to HED Based on Body Surface Area

Species	Reference Body Weight (kg)	Working Weight Range[a] (kg)	Body Surface Area (m²)	To Convert Dose in mg/kg to Dose in mg/m² Multiply by km below	To Convert Animal Dose in mg/kg to HED[b] in mg/kg, either	
					Divide Animal Dose by	Multiply Animal Dose by
Human	60	—	1.62	37	—	—
Child[c]	20	—	0.80	25	—	—
Mouse	0.020	0.011–0.034	0.007	3	12.3	0.081
Hamster	0.080	0.047–0.157	0.016	5	7.4	0.135
Rat	0.150	0.080–0.270	0.025	6	6.2	0.162
Ferret	0.300	0.160–0.540	0.043	7	5.3	0.189
Guinea pig	0.400	0.208–0.700	0.05	8	4.6	0.216
Rabbit	1.8	0.9–3.0	0.15	12	3.1	0.324
Dog	10	5–17	0.50	20	1.8	0.541
Primates						
Monkeys[c,d]	3	1.4–4.9	0.25	12	3.1	0.324
Marmoset	350	0.140–0.720	0.06	6	6.2	0.162
Squirrel monkey	600	0.290–0.970	0.09	7	5.3	0.189
Baboon	12	7–23	0.60	20	1.8	0.541
Micropig	20	10–33	0.74	27	1.4	0.730
Minipig	40	25–64	1.14	35	1.1	0.946

[a] For animal weights within the specified ranges, the HED for a 60 kg human calculated using the standard km value will not vary more than ±20% from the HED calculated using a km based on the exact animal weight.
[b] Assumes 60 kg human. For species not listed or for weights outside the standard ranges, HED can be calculated from the formula: HED = animal dose in mg/kg × (animal weight in kg/human weight in kg)$^{0.33}$.
[c] The km is provided for reference only since healthy children will rarely be volunteers for Phase 1 trials.
[d] For example, cynomolgus, rhesus, stumptail.

surface area determined experimentally by various methods. Compilations of this type of data can be found in published references.

For animal weights outside the working weight range in Table 16.6, or for species not included in the table, an alternative method is available for calculating the HED. In these cases, the following formula can be used:

$$\text{HED} = \text{animal dose (mg/kg)} \times [\text{animal weight (kg)/human weight (kg)}]^{0.33}$$

For example, assume that NOAEL of 25 mg/kg was determined in a study using rabbits weighing 4.0 kg. The 4.0 kg animals are outside the working range for rabbits of 0.9–3.0 kg indicated in Table 16.3.

$$\text{HED} = 25 \text{ mg/kg} \times (4.0/60)^{0.33} = 25 \times (0.41) = 10 \text{ mg/kg}$$

Alternatively, if the standard conversion factor was used to calculate the HED

$$\text{HED} = 25 \text{ mg/kg}/3.1 = 8.1 \text{ mg/kg}$$

The value of 10 mg/kg for the HED is 25% greater than the value of 8.1 mg/kg that would be calculated using the standard conversion factor.

The km analysis addresses only half of the HED conversion process. The range of human sizes must also be considered to convert the mg/m^2 dose back to an HED dose in mg/kg. To examine the effect of both animal and human weights on the conversion factor, the principle of allometry was used. Interspecies biologic parameters are often related by the power function Y = aW^b where W is body weight and b (allometric exponent) is the slope of the log–log plot, logy = bxlogW + C.

HED in mg/kg, this equation is

$$\text{(ii) HED} = \text{animal NOAEL} \times (W_{animal}/W_{human})^{(1-b)}$$

Since body surface area is believed to scale with an allometric exponent (b) of 0.67, one can explore how the animal and human body weights affect the conversion factor $(W_{animal}/W_{human})^{0.33}$.

The conversion factor was calculated over a range of animal weights and a range of human weights from 50 to 80 kg. The results are summarized in Table 16.7. Column B is the weight range of the animals used to calculate, in conjunction with the 50–80 kg range in humans, the conversion factor. The extremes of the conversion factors for the permutations chosen are shown in Columns C and D. The proposed standard conversion factors are shown in Column E. The percentage difference of these extremes from the standard is shown in Column F. Finally, the range of animal weights that produced a conversion factor for a 60 kg human within 20% of the standard factor is shown in Column G.

The conclusions from these analyses are as follows:

- The ±20 percent interval around the standard conversion factor includes a broad range of animal and human weights.
- Given that the human weights will vary broadly, it is not usually necessary to be concerned about the impact of the variation of animal weights within a species on the HED calculation.
- If an extreme animal weight is encountered in a toxicology study, one can calculate an accurate conversion factor using $(W_{animal}/W_{human})^{0.33}$.

The best scaling factor is not generally agreed upon. Though the majority opinion is that surface area is preferable where a metabolic activation or deactivation is known to be both critical to the adverse effect-causing process and present in both humans and the model species, these assumptions may not always be valid. And for the conditions under which most toxicity testing is performed, these facts are generally unknown. Table 16.8 presents a comparison of the weight and surface area extrapolation methods for the eight common laboratory animal species and humans. Brown et al. (1988), on reviewing the currently available data, likewise have concluded

Table 16.7   Effect of Body Weight on HED Conversions[a]

Species	Animal Weight Range[b] (kg)	Conversion Factor[c]			% Difference[e] of Extreme from Standard	±20% range[f] for 60 kg Human (kg)
		sm Animal lg Human	lg Animalsm Human	Standard[d]		
Mouse	0.018–0.033	0.060	0.089	0.081	–22%	0.015–0.051
Rat	0.090–0.400	0.106	0.213	0.162	–35%	0.123–0.420
Rabbit	1.5–3.0	0.269	0.395	0.324	+22%	1.0–3.4
Monkey	1.5–4.0	0.319	0.435	0.324	+34%	1.0–3.4
Dog	6.5–13.0	0.437	0.641	0.541	–19%	4.7–16.2

[a] Conversion factor = $(W_{animal}/W_{human})^{0.33}$.
[b] Human weight range used was 50–80 kg (110–176 lb).
[c] HED in mg/kg equals animal dose in mg/kg multiplied by this value.
[d] See standard table.
[e] Extreme from Column C or D.
[f] Range of animal weights that produced a calculated conversion factor within 20% of the standard factor (Column E) when human weight was set at 60 kg.

Table 16.8   Extrapolation of a Dose of 100 mg/kg in the Mouse to Other Species

Species	Weight (g)	Surface Area[a] (cm²)	Extrapolated Dose Based on (mg)		
			Body Weight (A)	Body Surface Area (B)	Ratio A/B
Mouse	20	46.4	2	2	1.0
Rat	400	516.7	40	22.3	1.80
Hamster	50	126.5	5	5.4	1.08
Guinea pig	400	564.5	40	24.3	1.65
Ferret	500	753.9	50	32.5	1.54
Rabbit	1500	1272.0	150	54.8	2.74
Dog	12000	5766.0	1200	248.5	4.82
Monkey	4000	2975.0	400	128.2	3.12
Man	70000	18000.0	7000	775.8	9.8

Note: Surface area (cm²) = $KW^{2/3}$, where K is a constant for each species and W is the body weight (values of K and the surface area for man are taken from Spector, 1956).

[a] Surface area (except in the case of man) values calculated from the formula:

that though "correlations exist among risk levels in various species, many factors appear to influence toxicity that are not captured in a simple scaling rule." These factors are commonly those pointed out in Table 16.4.

Schneiderman et al. (1975) and Dixon (1976) have published comparisons of these methods, but Schmidit-Nielsen (1984) should still be considered the primary source on scaling in interspecies comparisons.

When one is concerned about specific target organ effects, frequently the earliest indicator of such an effect is an alteration in organ weight out of proportion to what is to be expected due to changes in overall body weight (Gad et al., 1984) or to changes in a standard such as brain weight. It should be pointed out that a form of scaling is involved in detecting such effects, as adjustments to organ weight to account for alterations in overall body weight can take several forms. Either simple ratios (Angervall and Carlstrom, 1963; Gad and Weil, 1980; Weil and Gad, 1980), or analysis of covariance, or species or organ-specific allometric methods (Lutzen et al., 1976; Trieb et al., 1976) may be employed.

An alternative approach to achieving society's objective for the entire risk assessment process (that is, protecting the human population from unacceptable levels of voluntary risk) is the classic approach of using safety factors. This is still the methodology used in determining what are acceptable risks, given the uncertainties involved, in Phase I human clinical trials of a new drug would be based on animal safety data. The presumed degree of uncertainty in these cases is instructive. In 1972, Weil summarized this approach as follows:

> In summary, for the evaluation of safety for man, it is necessary to: (1) design and conduct appropriate toxicologically tests, (2) statistically compare the data from treated and control animals, (3) delineate the minimum effect and maximum no ill-effect levels (NIEL) for these animals, and (4) if the material is to be used, apply an appropriate safety factor, e.g., (a) I/100 (NIEL) or 1/50 (NIEL) for some effects or (b) 1/500 (NIEL), if the effect was a significant increase in cancer in an appropriate test.

This approach has served society reasonably well over the years, once the experimental work has identified the potential hazards and quantitated the observable dose–response relationships. The safety factor approach has not generally been accepted or seriously entertained by regulatory agencies for carcinogens, mutagens, or teratogen but is well established for other toxic effects of drugs and chemicals (Weil, 1972), and Johnson (1988) has recently, after reviewing a broad range of developmental and maternal toxicity data, proposed that "where the effect in the embryo is only seen at

maternally toxic doses and exposure is below the adult toxic doses, relatively modest safety factors are sufficient for safe cross-species extrapolation." Until such time as the more elegant risk assessment procedures can instill greater public and scientific confidence, the use of the safety factor approach to bridge our collective uncertainty about the difference between species responses should perhaps not be abandoned so readily for more "mathematically precise and elegant" procedures, but rather should be reviewed and perhaps revised for some areas of greater uncertainty (Dawson and Stara, 1983). Indeed, the FDA has returned to this approach as part of the guidance as to safe starting doses for clinical trials (FDA, 2002).

Once the HED of the NOAEL in the most appropriate species has been determined, a safety factor is then applied in order to provide a margin of safety for protection of human subjects receiving the initial clinical dose. This safety factor allows for variability in extrapolating from animal toxicity studies to studies in humans resulting from (1) uncertainties due to enhanced sensitivity to therapeutic activity in humans versus animals, (2) difficulties in detecting certain toxicities in animals (e.g., headache, myalgias, mental disturbances), (3) differences in receptor densities or affinities, (4) unexpected toxicities, and (5) interspecies differences in ADME of the therapeutic. These differences may be accommodated by lowering the human starting dose from the HED of the selected species NOAEL.

In practice, the maximum safe starting dose for the clinical trial is determined by dividing the HED derived from the animal NOAEL by the safety factor. The default safety factor used is 10. This is a historically accepted value, but, as described in the following text, should be evaluated based on available information.

While a safety factor of 10 can generally be considered adequate for protection of human subjects participating in initial clinical trials, this safety factor may not be appropriate for all cases. The safety factor should be raised when there is a reason for increased concern, and lowered when concern is reduced due to available data that provide added assurance of safety. This can be visualized as a sliding scale, balancing findings that mitigate the concern for harm to healthy volunteers with those that suggest greater concern is warranted. The extent of the increase or decrease is largely a matter of judgment, using the available information. It is incumbent on the evaluator to clearly explain the reasoning behind the applied safety factor when it differs from the default value of 10, particularly if it is less than 10.

Increasing the Safety Factor

The following considerations indicate a safety concern that might warrant increasing the safety factor. In these circumstances, the maximum recommended starting dose (MRSD) would be calculated by dividing the HED by a safety factor that is greater than 10. If any of the following concerns are defined in review of the nonclinical safety database, an increase in the safety factor may be called for. If multiple concerns are identified, then the safety factor should be increased accordingly.

Steep dose–response curve. A steep dose–response curve for significant toxicities in the most appropriate species or in multiple species may indicate a greater risk to the humans.

Severe toxicities. Qualitatively severe toxicities or damage to an organ system (e.g., central nervous system) indicate increased risk to humans.

Nonmonitorable toxicity. Nonmonitorable toxicities may include histopathologic changes in animals that are not readily monitored by clinical pathology markers.

Toxicities without prodromal indicators. If the onset of significant toxicities is not reliably associated with premonitory signs in animals, it may be difficult to know when toxic doses are approached in human trials.

Variable bioavailability. Widely divergent bioavailability in the several species, with poor bioavailability in the test species used to derive the HED, suggest a greater possibility for underestimating the toxicity in humans.

Irreversible toxicity. Irreversible toxicities in animals suggest the possibility of permanent injury in human trial participants.

Unexplained mortality. Mortality that is not predicted by other parameters raises the level of concern.

Large variability in doses or AUC levels eliciting effect. When doses or exposure levels that produce a toxic effect differ greatly across species, the ability to predict a toxic level in humans is reduced and a greater safety factor may be called for.

Questionable study design or conduct. Poor study design or conduct casts doubt on the accuracy of the conclusions drawn from the data. For instance, few dose levels, wide dosing intervals, or large differences in responses between animals within dosing groups may make it difficult to characterize the dose–response curve.

Novel therapeutic targets. Therapeutic targets that have not been previously clinically evaluated may increase the uncertainty of relying on the nonclinical data to support a safe starting dose in humans.

Animal models with limited utility. Some classes of therapeutic biologics may have very limited interspecies cross-reactivity or pronounced immunogenicity or may work by mechanisms that are not known to be conserved between (nonhuman) animals and humans; in these cases, safety data from any animal studies may be very limited in scope and interpretability.

Decreasing the Safety Factor

Safety factors of less than 10 may be appropriate under some conditions. The toxicologic testing in these cases should be of the highest caliber in both conduct and design. Most of the time, candidate therapeutics for this approach would be members of a well-characterized class. Within the class, the therapeutics should be administered by the same route, schedule, and duration of administration; should have a similar metabolic profile and bioavailability; and should have similar toxicity profiles across all the species tested including humans. A smaller safety factor might also be used when toxicities produced by the therapeutics are easily monitored, reversible, predictable, and exhibit a moderate-to-shallow dose–response relationship with toxicities that are consistent across the tested species (both qualitatively and with respect to appropriately scaled dose and exposure).

An additional factor that could suggest a safety factor smaller than 10 would be a case where the NOAEL was determined based on toxicity studies of longer duration compared to the proposed clinical schedule in healthy volunteers. In this case, a greater margin of safety is often built into the NOAEL, as it was associated with a longer duration of exposure than that proposed in clinical setting. This assumes that toxicities are cumulative, are not associated with acute peaks in therapeutic concentration (e.g., hypotension), and did not occur early in the repeat dose study.

As a final sanity check to any multistep process of hazard assessment, the data points generated by any other studies (particularly any human exposures) of the end point of interest should be evaluated to determine if they fall within the range expected based upon the assessment. If we find that the available real-world data does not fit our extrapolation model at this point, then as scientists we have no choice but to reject such a model or assessment and start anew.

Embodied in the safety factor approach are two of the "rules" for cross-species extrapolation, which are actually general comparative statements of relationships between species.

In general, as animal species become larger, they also become more sensitive to short-term toxicities. This effect may be credited to a number of mechanisms (such as increases of available target tissues and decreases of metabolic rate as size increases), but it is true even for nonmammalian species such as fish (Anderson and Weber, 1975) and birds (Hudson et al., 1979). The rule even applies somewhat to differences in body size within the same age class of the same sex of the same species.

Table 16.9 Some Relations of Drug Toxicity in Experimental Animals Compared to Humans

Species	Weight (kg)	Weight Ratio Animal/Human	Drug Dose Ratio	Sensitivity: Drug Dose Ratio/ Weight Ratio
Man	60	1	1	1
Cow	500	8	24	Human 3 × as sensitive
Horse	500	8	16	Human 2 × as sensitive
Sheep	60	1	3	Human 3 × as sensitive
Goat	60	1	3	Human 3 × as sensitive
Swine	60	1	2	Human 2 × as sensitive
Dog	10	1/6	1	Human 6 × as sensitive
Cat	3	1/20	1/2	Human 10 × as sensitive
Rat	0.4	1/150	1/15	Human 10 × as sensitive

The values in this table are averages and their validity cannot be checked against original data as Lehman (1959) only reported them as being from numerous sources.

What this means is that as a rule of thumb, the sensitivity of a larger species (such as a dog or human) to a short-term toxicity will be greater than that of a smaller species (such as a mouse or rat). There are, of course, exceptions and wide variations from linearity in this relationship (such as hamsters being much less sensitive to the neurotoxicity of DDT than mice, as reported by Gingell and Wallcave in 1974). And those toxicities that are mediated or modulated by structurally different features (such as those toxicities associated with the skin, where general rules fall completely apart across broad ranges of structural classes; Campbell and Bruce, 1981; Nixon et al., 1975) are subject to even less certainty under this rule.

There are also those who believe that humans are more sensitive than any test species, even if that species is larger than humans (such as a cow or horse). Lehman (1959) published, for example, the relationships shown in Table 16.9.

It should be noted that much data on effects in humans are biased by humans being better (or at least more sensitive and louder) indicators of adverse effects. Differences in sensitivity between the sexes are, in the majority, such that females are more sensitive than males. Data to support this will be reviewed later in this chapter.

SPECIAL CASES: MATCHING CHARACTERISTICS FOR SPECIAL POPULATIONS

There are many factors that can alter the physiological state of an individual (or the fraction of available chemical moiety in an individual, see Table 16.10), and in so doing make them more (or, in some few cases, less) susceptible to the adverse effects of a chemical. These include (but are not limited to) immunological experience, physiological factors such as stress, age, illness, conditioning factors (such as obesity and malnutrition), and sex. Proper model selection and experimental design require that these factors be identified and considered. Chapter 14 (susceptibility factors) will consider these and related factors on a theoretical basis in more detail.

Table 16.10 Factors That May Increase the Fraction of Available Chemical Moiety in the Systemic Circulation

1. Renal impairment
2. Liver impairment
3. Hypoalbuminemia
4. Presence of other moieties that displace test agent from proteins in circulation
5. Pregnancy

Sex

Sex hormones may be the target or may modify a particular toxic response that then may account for differential responses between the sexes to toxic materials. The current consensus is that (as was pointed out earlier) females are more susceptible than males to the acute toxic effects of many chemicals, though males and females of the same strain, age, and general condition will react in a qualitatively similar manner.

As a result of reviewing the acute oral and dermal toxicities of 98 pesticides to rats, Gaines (1969) concluded that by the oral route the majority were more toxic to females than to males. He found the reverse true for only 9 out of the 98 pesticides tested: aldrin, chlordane, heptachlor, abate, imidan, methyl parathion, fenchlorphos, schradan, and metepa. Pallotta et al. (1962) found the same pattern for the antibiotic acetoxycycloheximide in rats but not in dogs (where there was no sex difference). Indeed, a review of the published literature on pesticides by Kato and Gillete (1965) found that such sex differences are common in rodents but less so in other mammals, though the information on these other species is not as definitive. Even with rodents and pesticides, however, it should be remembered that this is a general rule and not a universal truth. Steen et al. (1976) found mevinphos to be more toxic to male Mongolian gerbils than to females, whereas Gaines (1960) found the reverse to be the case for this compound in rats. These observations were in accord with published data on hexobarbitone sleeping times, as shown in Table 16.11. In general, most barbiturates cause longer sleeping times in females than in males. Likewise, as a class, organophosphates are lethal in lower doses in female rats than in males.

Shanor et al. (1961) found that in humans there is a statistically significant difference between the plasma cholinesterase levels of healthy young males and females (activity levels in females are from 64% to 74% of males), but this difference disappeared in older people. There was no significant variation between the sexes in erythrocyte cholinesterase—a finding confirmed by Eben and Pilz (1967). Naik et al. (1970) likewise found there to be no significant difference between males and females in either total brain cholinesterase or in brain acetylcholine. These findings suggest that the distribution characteristics of toxicants working by cholinesterase inhibition mechanisms may be critical to their acute toxicity and that these distribution characteristics may be altered by the sex of the animal.

Krasovskij (1975) reviewed data on the acute toxicities of 149 chemicals and compared the results for males versus those for females. For both rats and mice, he found that the females tended to be more sensitive than the males, though not by large amounts (generally the differences were on the order of from 8% to 12%, being a little greater in rats than in mice).

Depass et al. (1984) looked at oral and dermal lethality of a number of previously studied compound on which the results had largely been published. To assess the effect of sex, they calculated the correlation coefficient (r) between the lethal dose parameters results for the two sexes. For 91 oral studies, r was found to be 0.93, whereas for 17 dermal studies with skin abrasion and 28 without, the r values were 0.73 and 0.96, respectively. The LD_{50} values between the two sexes were, in other

Table 16.11   Relative Hexobarbitone Sleeping Times for Each Sex in Two Different Rodent Species

Sex	Mean Sleeping Time (min)		
	Mongolian Gerbil[a]	Rat[b]	Mouse[c]
Male	105 + 9.6	22 + 4	34 + 5
Female	70 + 6.9	67 + 15	31 + 5

Note: The reported sex difference did not occur in rats less than 4 weeks old.

[a] Maines and Westfall (1971).
[b] Quinn et al. (1958).
[c] Vessell (1968).

words, strongly associated. However, when the values were compared using paired t-tests, there was a statistically significant trend toward higher LD_{50}s in the males.

Similarly, Bruce (1985) reviewed studies from files on 48 chemicals and found that for only three of these there was evidence of lower LD_{50} values among the males than the females, and that none of these differences approached statistical significance. In 13 cases, however, the males had significantly higher LD_{50} values than the females. For these 13 studies, male LDP values averaged 29% higher than those for females.

Imbalances of hormones other than those related to sexual function have also been shown to alter the susceptibility of animals to the toxic effects of chemicals. Hyperthyroidism, hyperinsulinism, adrenalectomy, and stimulation of the pituitary have all been demonstrated to be capable of modifying the effects of selected toxicants (Dauterman, 1980; Doull, 1980).

Stress

Stress and the variability of animals underlying biological rhythms (the complex interactions of physiological responses to chronologically ordered external factors) are among the least accounted for valuables in toxicology. Though they have both been studied and identified as important determinants of sensitivity to toxicity, standard practice does not evaluate these effects or seek to factor them in predicting human effects.

Stress is a broad term for specific morphological, biochemical, physiological, and/or behavioral changes experienced by an organism in response to a stressful event or "stressor" (Vogel, 1987). Such changes can be quite drastic. Plasma levels of epinephrine in resting rats are approximately 100 pg/mL, but in a stressed rat, it can approach 2000 pg/mL. Cessation of the stressful event usually terminates the stress response, and the organism returns to its baseline homeostasis. However, if the stress response is very intense or long lasting, a return to the original homeostasis may not occur and a new biological equilibrium may be established. The consequences of this new condition can be either beneficially (such as exercise induced stress strengthening the heart) or detrimental (such as job induced stress causing ulcers or hypertension) to the organism.

The typical behavioral stress responses are fear, tension, apprehension, and anxiety. Physiological stress responses can include changes in gastric secretion and motility and increases in blood pressure and heart rate. Biochemical changes are widespread during stress and include significant increases in the levels of plasma catecholamine and corticosteroids or marked changes in brain neurotransmitter concentrations. Although these are only typical response examples, most biochemical and physiological systems are probably affected during stress. Thus, potential toxicants interact with quite different physiological and/or biochemical systems during stress, and the resulting outcomes of such interactions are bound to be quite different under these altered conditions.

In experimental toxicology, it is customary to use nonstressed animals to evaluate the extent and modes of action of chemicals. However, animals and humans are seldom nonstressed, but rather are frequently challenged by stressful events in the real world, responding with stress manifested as some of the previously mentioned responses. Thus, agents acting at specific biochemical sites or on physiological processes will encounter different conditions during rest and stress, resulting in differences in their effects. In addition, the true action of some agents may only be revealed during stress. Thus, the variable of stress should probably be included during experimentation to better approximate (or model) various real-life situations and to predict more accurately the actions of chemicals under all types of environmental conditions.

The literature does very clearly reflect that the actions of biologically active substances can be altered during stress. Toxic effects can be increased or decreased, and the results must be interpreted in this context before they can be generalized or extrapolated to the human population. Guinea pigs show an increased susceptibility to the lethal effects of ouabain during stress.

Natelson et al. (1979) report that only 9% of nonstressed animals die, whereas 50% succumb to the same dose of ouabain if the animals are stressed.

Similarly, the delayed neuropathology to triorthotolyl phosphate in hens is almost tripled by stress (Enrich and Gross, 1983), and Stockinger (1953) indicated the considerable influence that stress can have on the dose-dependent distribution of some of the elements in the body.

It has also been demonstrated that injections of adrenocorticotropic hormone (Vaccarezza and Wilson, 1964a,b) caused increases both in plasma and cell cholinesterase in rats, but that adrenalectomy caused a progressive decline in the circulating RBC cholinesterase but had no effect on plasma cholinesterase in rats. In people, injections of adrenocorticotropic hormone also gave rise to increases in plasma and circulating cell cholinesterase (Vaccarezza and Pelts, 1960).

In reporting investigations of parathion in rats, Kling and Long (1969) demonstrated the influence that dietary stress could have on the time course of the response of cellular cholinesterase, while not altering the overall quantitative outcome. In fact, much of life (for at least the laboratory animals species) consists of a habitat that exhibits a recurrence of a sequence of events in an ordered manner relative to time. The effect of many biologically active agents, particularly toxicants, must interfere with these normal patterns and resulting biological cybernetics. Though Scheving et al. (1974) have held that there was little evidence that acute toxicity displayed significant differences relative to circadian rhythms, this conclusion seems suspect. There are clear relationships between biorhythms, stress, and the endocrine functions.

Circadian differences in response to a range of chemicals such as nikethamide, ethanol, chlordiazepoxide (Librium), methopyrapone, and ouabain have been observed in the mouse. Halberg et al. (1960) demonstrated that there was a potency ratio alteration of from 3.2 to 1.0 for the bioassay of *Escherichia coli* endotoxin carried out at 12 hour time intervals. And working with rats, Lenox and Frazier (1972) demonstrated that the mortality due to methadone was influenced by a circadian cycle.

Stress due to fasting has been shown to alter the permeability of the blood–brain barrier to some chemicals (Angel, 1969). Indeed, selective starvation can also influence sensitivity—Boyd et al. (1970) demonstrated that feeding protein-deficient or protein-rich diets to rats could markedly alter the LD_{50} values for many pesticides.

Likewise, for some toxicants the influence of single or multiple housing can also significantly alter the results of a range of outcomes in acute toxicity tests, with marked variations in sensitivity (and the direction even of the influence) to this housing variable between different species.

Age

Age is an endogenous factor that alters an organism's response to exposure to a test chemical. Very young or old animals may be either more or less sensitive to toxic effects than fully developed young or mature animals and indeed may even have qualitatively different responses. Older rats, for example, are almost immune to the carcinogenic actions of most chemicals. Neonates are more susceptible to the actions of opiates.

Traditionally, what is used to perform tests in our studies are young adult animals. However, much of our human population is either very young or old and clearly not physiologically comparable to young adults.

Age variation may give rise to differences in susceptibility to acute intoxication by different chemicals, and there is not a simple rule for relating age to the sensitivity or nature of the toxic response. Goldenthal (1971) published an extensive review of the comparative acute toxicity of agents to newborn and adult animals. During the early stages of life, anatomical, physiological, metabolic, and immunological capabilities are not fully developed.

Substantial differences in susceptibility can sometimes even be related to small age differences. With rodents, a few months difference in age can markedly alter the response to chemicals that influence either the central nervous or immunological systems.

Biological aging is both time and species dependent (Mann, 1965). For the purposes of acute toxicity, it is generally convenient to consider the stages of biological age as being neonatal, infant, young adult, and old. There is no clear dividing line between these stages in any species, though their length was loosely defined at the beginning of this chapter. Rather, development and aging are a continuum on which there is both species and individual variation. For some laboratory-bred species, however, such as the rat, there is a fairly linear relationship between the logarithm of body weight and the reciprocal of the animal's age (Gray and Addis, 1948), which can be expressed as

$$\log_{10}[W] = \frac{-K}{d}a + \log_{10}A$$

where
   W is the weight in grams
   K is the slope
   d is the age in days
   A is the estimated asymptote or limit for W

There is in fact an entire family of statistical methods for adjusting different structural and functional characteristics for age. Mattfeldt and Mall (1987) give an excellent overview of these allometric methods.

The toxicological response to both exogenous and endogenous physiological chemicals (such as epinephrine and acetylcholine) can vary with age. Brus and Herman (1971) demonstrated that newborn mice were significantly less sensitive to epinephrine and norepinephrine than adults, but that the reverse held true for acetylcholine. Naik et al. (1970) found that brain acetylcholine concentrations increased with body weight/age until maturity, whereas brain cholinesterase activities were variable at lower weight/age and became less variable as weight/age increased. Shanor et al. (1961), using a large population sample of young adults (ages 18–35 years) and older people (ages 70–80 years), found that the plasma cholinesterase activity was approximately 24% higher in the young males than in the old males, but that no such difference existed for females or for RBC cholinesterase.

Older animals also show a large number of alterations in their response to potential toxicants when compared to young adults.

Disease

Disease states can modify a variety of kinetic and physiological parameters, altering the baseline homeostatic condition. Earlier, it was pointed out that a number of conditions (such as liver or renal disease) could increase the amount of available drug moiety in the systemic circulation. The ability to understand how pathological conditions can modify the kinetics and effect of exogenous chemicals requires an understanding of the interrelationships between these various parameters (Holmes, 1984).

Stress due to infection can alter the responses of animals to biologically active chemicals. In 1972, Safarov and Aleskerov found that the dipping of sheep in an ectoparasiticide depressed antibody production and reduced the ability of the sheep to survive infections. It has been shown that some chemicals adversely affect the natural immunological defense systems, although this appears to be associated more with persistent agents retained in the organism than with those agents that are rapidly cleared. Liver (by decreasing biotransformation) and renal (by disrupting both excretory and metabolic functions) diseases associated with a preexisting condition or old age may contribute to a greater sensitivity to a toxicant. Hyperthyroid states also have been shown to increase sensitivity to the toxic effects of several classes of drug, particularly selected psychoactive agents (Zbinden, 1963).

Physiological State

The influence of diet on the response of animals and humans to toxins is well established. The toxicity of specific agents can be increased or decreased by alterations of dietary protein or the various micronutrients (an example being the decreased sensitivity of protein-deficient animals to CCl[sub]4). Two conditions that occur in humans and are not generally recognized as diseases (obesity and subclinical malnutrition or marginal nutrition) can also alter the biological effects of chemicals and serve to increase the susceptibility of individuals to toxic actions.

Obesity may well alter the distribution and storage of a xenobiotic, especially when it is markedly lipophilic. Obesity is also generally accompanied in humans and test animals by reduced or impaired respiratory, cardiovascular, and renal function, all of which will also alter the manner and degree to which an agent may be toxic. Sleeping versus awake ratio will also have an influence (Brown, 1961).

Subclinical malnutrition or marginal nutrition is the other end of the scale from obesity usually, but not always or absolutely. An individual person's or animal's diet may be calorically adequate (or even oversupplied) but nutritionally marginal in terms of vitamins, proteins, minerals, and other nutrients. Any such marginal nutrient state clearly presents the possibility of an increased susceptibility to a toxic or adverse outcome of exposure to a xenobiotic, particularly if said nutritional state means limited or deficient metabolic and/or enzymatic defense mechanisms or if the potential toxicant acts to reduce the available limiting nutrient. The principal biotransformation of toxicants is performed by the microsomal mixed function oxidase system, which is depressed by a deficiency of essential fatty acids, vitamin A, or proteins.

Boyd et al. (1970), Boyd (1972) and Evans (1982) reviewed the effects of nutritional status on acute toxicity, showing alteration in the responses of rodents. While others (Ensminger and Olentine, 1978, in minks) have done the same in other species. Mehrle et al. (1973) demonstrated that the LC_{50}s of chlordane in rainbow trout were altered by the brand of commercial diet the fish were maintained on beforehand. Furthermore, the nutritional status of animals used to prepare or provide tissues for in vitro studies can change the microsomal metabolism and other aspects of responsiveness of the tissue (Kato and Gillette, 1965).

Toxins also have the potential to induce nutritional deficiencies, but these are generally of concern only in cases of longer term exposure.

MODELS

This entire volume has focused on animal models in toxicology and is based on the premise that these models are not only a valid approach to predicting the adverse effects of chemicals in humans, but are the primary model for such predictions.

In vivo models are not the only means, however, and a brief review of the status of alternatives to them is called for. Classes of in vitro alternatives are in vitro models (which do not use intact higher organisms but do use some forth of test system) and mathematical or SAR (structure activity relationship) approaches (which construct theoretical analogies but require no actual generation or interpretation of new data).

In Vitro Models

In vitro models, at least as screening tests, have been with us in toxicology for some 20 years now. The last 5–10 years have seen a great upsurge in interest in such models. The increased interest is due to economic and animal welfare pressures and technological improvements.

In vitro systems per se have a number of limitations that can contribute to their not being acceptable models. Some of these reasons are detailed in Table 16.12.

Table 16.12   Possible Interpretations When In Vitro Data Do Not Predict Results of In Vivo Studies

1. Chemical is not absorbed at all or is poorly absorbed in in vivo studies.

2. Chemical is well absorbed but is subject to first-pass effect in liver.

3. Chemical is distributed so that less (or more) reaches the receptors than would be predicted on the basis of its absorption.

4. Chemical is rapidly metabolized to an active or inactive metabolite that has a different profile of activity and/or different duration of action than the parent drug.

5. Chemical is rapidly eliminated (e.g., through secretory mechanisms).

6. Species of the two test systems used are different.

7. Experimental conditions of the in vitro and in vivo experiments differed and may have led to different effects than expected. These conditions include factors such as temperature or age, sex, and strain of animal.

8. Effects elicited in vitro and in vivo differ in characteristics.

9. Tests used to measure responses will probably differ greatly for in vitro and in vivo studies, and the types of data obtained may not be comparable.

10. The in vitro study did not use adequate controls (e.g., pH, vehicle used, volume of test agent given, samples taken from sham-operated animals).

11. In vitro data cannot predict the volume of distribution in central or in peripheral compartments.

12. In vitro data cannot predict the rate constants for chemical movement between compartments.

13. In vitro data cannot predict whether linear nonlinear kinetics will occur with specific chemical in vivo.

14. In vitro data cannot predict whether linear or nonlinear kinetics will occur with specific dose of a chemical in vivo.

15. Pharmacokinetic parameters (e.g., bioavailability, peak plasma concentration, half-life) cannot be predicted based solely on in vitro studies.

16. In vivo effects of chemical are due to an alteration in the higher order integration of an intact animal system, which cannot be reflected in a less complex system.

At the same time there are substantial potential advantages in using in vitro systems. The scientific advantages of using cell or tissue culture in toxicological testing are isolation of test cells or organ fragments from homeostatic and hormonal control, accurate dosing, and quantitation of results. It is important to devise a suitable model system that is related to the mode of toxicity of the compound. Tissue and cell culture have been used in two very different ways in screening studies. First, they have been used to examine a particular aspect of the toxicity of a compound in relation to its toxicity in vivo. Second, they have been used as a form of rapid screening to compare the toxicity of a group of compounds. Additionally, target organ-specific in vitro models offer a powerful means of explaining the mechanisms behind toxicities observed in intact organisms.

SUMMARY

If the human population we are concerned about is such that one or more of these susceptibility factors is present in a substantial portion of the members, steps should be taken to design studies so that such individuals are adequately represented (i.e., "matched") by an appropriate model in the test animal population. Barring that, or in the face of having existing data on studies performed in a standard manner, consideration should be given to these factors when attempting to predict outcome of exposures in people.

Individual Biological Variation

There are also individual animal-to-animal variations in temperature, health, and sensitivity to toxicities, which are recognized and expected by experienced animal researchers, but are only broadly understood. The resulting differences in response are generally accredited to "individual biological

variation." This same phenomenon has been widely studied and observed among humans and is expected by any experienced clinician. Examples of such individual variations in humans include isoniazid, succinylcholine, and glucose-6-phosphate levels and/or activities. In the first of these, "slow inactivators" who are deficient in acetyltransferase (and therefore acetylate agents such as isoniazid only slowly) are thus more liable to suffer from the peripheral neuropathy caused by an accumulation of isoniazid. At the same time, people with more effective acetyltransferase require larger doses of isoniazid to benefit from its therapeutic effects, but in so doing are more likely to suffer liver damage.

Likewise, individuals with atypical or low levels of serum cholinesterase may exhibit prolonged muscle relaxation and apnea following an injection of a standard dose of the muscle relaxant suc-cinylcholine. And glucose-6-phosphate dehydrogenase deficiency is responsible for the increased probability of some individuals given primaquine or antipyrine to suffer from a hemolytic anemia.

Species Variation

Though anyone who has had to work in biological research with intact animals should be aware of the existence of wide variability between species, examples which are specific to toxicology should be presented here along with some degree of comparison of relative species sensitivity for a number of specific agents. Table 16.13 presents an enumeration of target organ toxicities, which are specific to several model-specific species commonly used in toxicology.

The rodenticide zinc phosphide is dependent on the release of phosphine by hydrochloric acid in the stomach for its activation and efficacy (Johnson and Voss, 1952). As a result, dogs and cats are considerably less sensitive than rats and rabbits since the former species secrete gastric hydrochloric acid intermittently while the latter secrete it almost continuously.

That this case is not a rare one can be quickly established by examining some data sets where we have comparative oral lethality data on several species (including humans), such as those presented in Table 16.14.

Hottendorf has published a review of the predictive value of seven animal model species across a range of compounds with different target organs, finding the rat, mouse, and monkey to be generally the best individual predictors while the guinea pig and hamster were the least accu-rate predictors.

Table 16.13   Species-Specific Toxic Effects

Type of Toxicity	Structure	Sensitive Species	Mechanism of Toxicity
Ocular	Retina	Dog	Zinc chelation
Ocular	Retina	Any wit pigmented retinas	Melanin binding
Stimulated basal metabolism	Thyroid	Dog	Competition for plasma binding
Ocular	Retina and optic nerve	Primates	Formic acid formation
Porphyria	Liver	Human, rat, guinea pig, mouse, and rabbit	Estrogen-enhanced sensitivity
Tubular necrosis	Kidney	Rats (males)	Androgen-enhanced sensitivity[a]
Urolithiasis	Kidney and bladder	Rats and mice	Uricase inhibition
Teratogenesis, mortality	Fetus	Rats and mice	Uricase inhibition
Cardiovascular	Heart	Rabbits	Sensitivity to microvascular constriction

Sources: Adapted from Gralla, E. J., Species specific toxicoses with some underlying mechanisms, in *Safety Evaluation of Drugs and Chemicals*, Lloyd, W. E. (ed.), Hemisphere, New York, pp. 55–81, 1986, with modification; Baker, H. J. et al., *The Laboratory Rat. Vol. I.* Academic Press, New York, pp. 411–412, 1979.
[a] More sensitive than humans for many agents (such as caprolactam and halogenated solvents).

Table 16.14   Comparative Human Acute Lethal Doses and Animal LD_{50}s (mg/kg via Oral Route)

Chemical	Human LD_{LO}[a]	LD_{50} Values			
		Mouse	Rat	Rabbit	Dog
Aminopyrine	220	358	685	160	150
Aniline	350	300	440		195
Amytal	43	345	560	575	
Boric acid	640	3450	2660		
Caffeine	192	620	192	224	140
Carbofuran	11	2	5		19
Carbon tetrachloride	43	12800	2800	6380	
Cycloheximide		133	3		
Lindane	840		125	130	120
Fenoflurazole		1600	283	28	50

[a] LD_{LO} = lowest observed lethal dose.

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Susceptibility Factors

Shayne Cox Gad

CONTENTS

Table 17.1 Factors That May Increase the Fraction of Available Chemical Moiety in the Systemic Circulation (and Therefore Potential Toxicity)

1. Renal impairment
2. Hepatic impairment
3. Impaired gastrointestinal absorption
4. Hypoalbuminemia
5. Presence of other moieties that displace test agent from proteins in circulation
6. Pregnancy
7. Gender

Sources: Zbinden, G., Experimental and clinical aspects of drug toxicity, *in Advances in Pharmacology*, S. Garratini, and P. A. Shore, eds. Academic Press, New York, 1963; Dauterman, W. C. Physiological factors affecting metabolism of xenobiotics, in *Introduction to Biochemical Toxicology*, E. Hodgson and F. E. Guthrie, eds. Elsevier, New York, 1980; Doull, J., Factors influencing toxicology, in *Casarett and Doull's Toxicology*, J. Doull, C. D. Klaassen, and M. O. Amdur, eds. Macmillan, New York, 1980; Depass, L. R. et al., *Alternative Methods in Toxicology, Vol. 2 Acute Toxicity Testing*. Mary Ann Liebert, New York, pp. 141–153, 1984; Krasovskij, G. N. Species and sex differences in sensitivity to toxic substances, in *Methods Used in the USSR for Establishing Biologically Safe Levels of Toxic Substances*. World Health Organization, Geneva, Switzerland, 1975.

Susceptibility factors are conditions or characteristics that make individual animals or discrete groups of animals (or people) differentially more sensitive to the toxicity of an agent or to expression of manifestations of the toxicity. Such factors should be considered from two different perspectives. First, if undesired, they may serve to either confound the results of experiments or, by increasing the variability of response in text populations, decrease the sensitivity of test systems. Second, if our concern is the health and safety of more sensitive individuals in the population (as opposed to the young, healthy adult population whom our usual test animal pool represents), then incorporation of such factors in selecting model populations is essential. Unfortunately, this latter is something we still do not do well.

There are many factors that can alter the physiological state of an individual (or the fraction of available chemical moiety—see Table 17.1) and in so doing make them more (or, in some few cases, less) susceptible to the adverse effects of a chemical. These include (but are not limited to) immunological experience; psychological factors such as stress, age, and illness; conditioning factors (such as obesity and malnutrition); and genetic background and gender. There are also environmental factors such as temperature, humidity, and time of day that can serve as susceptibility factors. Even the physical size of administered doses can be an important factor influencing susceptibility (Stockinger, 1953).

As a starting place for examining the influence (and possible uses) of susceptibility factors, we should first consider the "base case." That is, what is the nature of the commonly used animal model?

DEFINED ANIMAL MODEL

Since at least the mid-1970s, it has become an accepted practice in toxicology to use bred-to-purpose, genetically defined (usually inbred), and specific pathogen-free (SPF) experimental animals. Such animals are most commonly obtained from one of four commercial vendors and put on study while they are young adults in the log phase of growth.

Festing (Strong, 1942; Festing, 1979a, 1981) in his review of the development of these animal stocks has called them "defined" animals in terms of their microbiological flora, genetic background, and environmental and nutritional care.

It must be noted that the influence of these is further confounded (or possibly accentuated) by age and gender. Postmarket approval drugs associated with patient adverse drug reactions (ADRs) that are believed to be predictable based on nonclinical study results are generally due to a number of factors. These are summarized in Table 17.2.

Table 17.2   Factors Predisposing to Pharmacological Type A ADRs (Susceptibility Factors)

Type	Example	Toxicity	Mechanism
Pharmaceutical	Phenytoin	Phenytoin toxicity (ataxia, nystagmus, etc.)	Increase in bioavailability because of a change in formulation
Pharmacokinetic (can involve absorption, distribution, metabolism, and excretion)	Digoxin	Digoxin toxicity (nausea, arrhythmias, etc.)	Decreased elimination if renal function is impaired
Pharmacodynamic	Indomethacin	Left ventricular failure	Water and sodium retention
Genetic	Nortriptyline	Confusion	Reduced hepatic elimination because of a deficiency of CYP2D6
Drug–drug interactions (can involve any of the aforementioned processes)	Lithium and nonsteroidal anti-inflammatory drugs	Lithium toxicity	Inhibition of excretion of lithium

Renal Impairment, Xenobiotic Metabolism, and Toxicity

It should not be surprising that there is special interest in patient populations in which there is compromised function of eliminating or clearing organs (Brater et al., 1992; Matzke and Milikin, 1992). The organs of particular interest are the kidney and liver, the primary clearing organs primarily responsible for drug elimination. Impairment of either of those organs has implications in terms of drug accumulation and potential toxicity. There is a need, therefore, to adjust dosing, especially for those drugs having a narrow therapeutic concentration range. The FDA issued a draft guidance "Pharmacokinetics and Pharmacodynamics in Patients with Impaired Renal Function: Study Design, Data Analysis, and Impact on Dosing and Labeling" in March 2010 on just this point.

It makes sense to examine the pharmacokinetics and pharmacodynamics of those xenobiotics primarily or substantially excreted unchanged via the kidney (e.g., lithium). It would also make sense to extend such studies to those compounds that are primarily metabolized by the liver but whose metabolites are predominantly excreted into the urine; the latter is a common pathway for many if not most metabolites. The concern here is with metabolites that are pharmacologically active and/or toxic, since such metabolites will accumulate in the presence of renal insufficiency (e.g., N-acetylprocainamide, the active metabolite of procainamide).

A further complication is that renal impairment is associated with other conditions that may have an impact on the pharmacokinetics and pharmacodynamics of the drug. These changes in, for example, pharmacokinetics, may be the result of indirect effects. For example, in some renal diseases, plasma proteins may be excreted into the urine, resulting in a lowering of plasma protein concentration. The latter may influence (i.e., decrease) plasma protein binding (f_u increases), which may, in turn, influence (i.e., increase, for low-clearance drugs) the elimination of the drug by other clearing mechanisms (e.g., hepatic). It is apparent that one needs to have a reasonably good understanding of the pharmacokinetics of the drug, especially route of elimination, plasma protein binding, and clearance, in order to decide whether or not to conduct studies in renal impaired subjects. The latter are not simple studies and they are expensive and time-consuming.

It is quite fortunate that we have a direct physiological measure of renal function that may be used in assessing the degree of renal insufficiency and that may be used quantitatively to alter drug dosing regimens. That measure is creatinine clearance (CLCr). Creatinine is an end product of muscle metabolism that is exclusively excreted via the kidney. While passive glomerular filtration appears to be the predominant excretory pathway, there have been some suggestions of active secretion. In either event, the compound serves as a good marker of renal function and it has an additional advantage; it is produced endogenously. As a consequence, it is not necessary to administer a test compound (e.g., inulin,

a high-molecular-weight polysaccharide that is cleared exclusively by glomerular filtration). CLCr may be measured experimentally by obtaining a complete (and it must be complete) urine collection, generally for 24 hours, and a single blood sample to determine serum creatinine concentration. The latter is often determined at the beginning or middle of the urine collection period, and one assumes that the serum concentration remains essentially constant during that time, generally, a good assumption. One caution here is that some cooked meats (especially boiled beef) contain creatinine that, following absorption, will increase urine creatinine excretion and result in an overestimate of CLCr (assuming that the serum creatinine concentration does not reflect this absorption).

It is unusual to actually measure CLCr in the subject, for a variety of reasons including inconvenience, errors in urine collection, etc. It is more usual that we estimate renal function, CLCr, from a measure of serum creatinine concentration (SCR). The latter is quite convenient since it represents one of the many biochemical measures routinely determined in a battery of blood chemistries. However, CLCr is not directly related to SCR and we need to rely upon a relationship between the two that has been determined in studies of relatively large number of subjects. Such a relationship exists in several very similar forms often referred to as the Cockcroft–Gault and Siersback–Nielsen relationships or equations. The Cockcroft–Gault equation is based upon a study of about 200 subjects with varying degrees of renal function.

$$\text{CLCr}\left(\text{mL}/\text{min}\right)_{\text{MALE}} = \frac{\left(140\text{-age}\left(\text{year}\right) \cdot \text{Weight}\left(\text{kg}\right)\right)}{72 \cdot \text{SCR}\left(\text{mg\%}\right)}$$

It is important that the correct units be used: age in years, weight (as lean or ideal weight) in kg, and SCR in mg%. The resulting clearance will have units of ml/min. The preceding equation is appropriate for males. For females, the equation needs to be adjusted downward by multiplying by 0.85. The latter is on the basis of a smaller muscle mass in females:

$$\text{CLCr}\left(\text{mL}/\text{min}\right)_{\text{MALE}} = \frac{\left(140\text{-age}\left(\text{year}\right) \cdot \text{Weight}\left(\text{kg}\right)\right)}{72 \cdot \text{SCR}\left(\text{mg\%}\right)}$$

There remains some controversy concerning which weight to use: lean or ideal weights. The latter is a special problem in obese subjects. If the actual weight is less than ideal, use the actual weight. Some investigators use the following equations for estimating ideal body weight (IBW) in males and females:

$$\text{IBW}_{\text{MALE}}\left(\text{kg}\right) = 50 + \left[2.3 \times \left(\text{height in inches} - 60\right)\right]$$

$$\text{IBW}_{\text{FEMALE}}\left(\text{kg}\right) = 45.5 + \left[2.3 \times \left(\text{height in inches} - 60\right)\right]$$

Serum creatinine concentration and creatinine clearance are related in a nonlinear fashion. A similar nonlinear relationship is also seen between the elimination half-life of a drug and CLCr.

A linear relationship will exist between K or CL of the drug and CLCr, and it is those relationships that are most often used in adjusting a dosing regimen. The following relationship would be expected:

$$K = m \cdot \text{CLCr} + k_{\text{NR}} \quad K = m \cdot \text{CLCr} + \text{CL}_{\text{NR}}$$

The relationships noted earlier provide a basis for the estimation of K or CL in a given subject. A value of SCR is obtained, and from that, an estimate of CLCr is calculated using the aforementioned equations (or nomogram) noted. The value for CLCr is then entered into one of the preceding relationships to estimate K or CL, and from that, a value of half-life is obtained. The information is then used to calculate an individualized dosing regimen for the subject with renal impairment. Alternatively, one

can locate tables that contain values for K or CL for normal and anephric subjects (and corresponding values for CLCr). From those two values, one can estimate a slope and intercept and use that information to estimate K or CL in the subject of interest whose value for CLCr has been estimated.

The more substantial the renal excretory process relative to all pathways of elimination (i.e., the larger the ratio, CL_R/CL_s), the greater the impact of renal insufficiency is on the pharmacokinetics of the drug.

There are several complications that need to be considered here with regard to the influence of renal impairment on the pharmacokinetics of drugs. These considerations are in addition to the obvious direct effect of renal impairment on the elimination of the parent drug, as discussed earlier.

One consideration is the accumulation of active or toxic metabolites of the parent drug that would normally be excreted via the kidney. Such metabolites will accumulate in renal insufficiency since they will be renally cleared less efficiently. Examples include the following parent and metabolite pairs:

Parent Drug	Metabolite
Allopurinol	Oxipurinol
Cilazapril	Cilazaprilat
Meperidine	Normeperidine
Primidone	Phenobarbital
Procainamide	N-acetylprocainamide
Propoxyphene	Norpropoxyphene
Ofloxacin	Desmethylofloxacin

The dosing regimen of the parent drug may need to be reduced as a consequence of the accumulation of the active metabolite.

An additional complication is the alteration in the plasma protein binding of drugs as a result of two mechanisms. During renal insufficiency, endogenous waste materials will accumulate and such compounds have the potential to compete with drugs for protein binding sites (e.g., fatty acids). Plasma protein concentrations may be reduced in association with renal diseases such as uremia and nephrotic syndrome. A reduction in protein concentration will result in a decrease in the number of binding sites, and this may result in reduced drug binding. The implications of this effect will depend upon the drug, especially its value of clearance. A good example is phenytoin, which is highly bound to albumin and is completely metabolized by the liver. If the drug has a low (restrictive) clearance, such as phenytoin, f_u will increase and there will be a corresponding increase in clearance since $CL \approx f_u \cdot CL_{u,i}$. Most importantly, however, there will be no change in the unbound concentration of phenytoin, even though the total concentration declines:

$$C_T\downarrow = \frac{\text{dose rate}}{\uparrow f_u \cdot CL_{u,i}}$$

$$C_T\leftrightarrow = \frac{\text{dose rate}}{CL_{u,i}}$$

Alteration in plasma protein binding would suggest that the apparent volume of distribution may increase or be minimally changed. There is at least one drug example of a decrease in apparent volume of distribution in renal insufficiency, digoxin. Digoxin has a very large apparent volume of distribution (ca. 10 L/kg), indicating that most of the drug is outside of the vascular region. The drug binds extensively to muscle tissue. It appears that the latter binding process is altered during renal insufficiency, resulting in digoxin movement from the tissue into the blood. The latter results in a smaller apparent volume of distribution. The only clinical significance that this has is in the calculation of a loading dose for the drug (it must be reduced).

A somewhat more complex and far more limited situation has been referred to as "futile metabolism." In this instance, the metabolite formed is unable to be excreted into the urine because of renal insufficiency. However, if the metabolite can undergo reversible metabolism to the parent drug, the drug will have a prolonged residence time in the body; the metabolic step is "futile" in terms of getting rid of the substance. An example appears to be acyl-glucuronide metabolites (e.g., those of clofibrate, diflunisal, and some arylpropionic nonsteroidal anti-inflammatory agents). The paradox is the observation that the parent drug, which undergoes metabolism, accumulates in the presence of renal insufficiency, even though metabolic function has not been altered.

The FDA Guidance offers several possible study designs depending upon the characteristics of the drug under study. A full study is recommended if the drug is excreted primarily by the kidney. There are four groups to be studied. The control group is the typical patient population or equivalent. A normal healthy group can be included but cannot replace the control group. The study design can be single dose (if linear kinetics) or multiple dose (if nonlinear kinetics). A concentration-controlled study is also acceptable. Unbound and total plasma concentrations should be determined (if highly bound) and CLCr should be measured. A sufficient number of subjects should be selected to determine if there is a pharmacokinetic difference necessitating dose adjustment. The following groups are suggested:

Group	Renal Impairment	% Normal Renal Function
1	Controls	>80%
2	Mild	40%–80%
3	Moderate	10%–40%
4	Severe	<10%

Alternatively, it might be acceptable to perform a reduced study in only two groups (groups 1 and 4). If no pharmacokinetic differences are found, no further study is necessary. A population-based pharmacokinetic study might also suffice, if there is a sufficiently wide range of renal function within the population.

A study in hemodialysis or peritoneal dialysis patients would not be necessary if the drug has an unbound apparent volume of distribution greater than about 350 L. Drugs of the latter ilk undergo very poor dialysis (i.e., less than about 10% removal during typical dialysis).

Renal excretion changes dramatically with aging. Glomerular filtration as measured by inulin clearance (1) decreases with age as does creatinine clearance (not shown). Tubular excretory capacity (2), renal blood flow (3), and renal plasma flow (4) all decline with age. Interestingly, if all of those values are plotted as a percentage of the value at age 20–29, the lines almost superimpose. This suggests that all aspects of renal function decline in parallel with age in the absence of disease states.

Some care needs to be taken in interpreting serum creatinine clearance in the elderly and its relationship with creatinine clearance, the latter being the better estimate of renal function. Serum creatinine concentration remains relatively constant with age (in the absence of renal disease). The following equation relates steady-state creatinine serum concentration to creatinine clearance, and as with all similar steady-state equations, it is a function of rate in or production rate (numerator) and rate out or clearance (denominator):

$$\text{Serum creatinine concentration} = \text{SCR} = \frac{\text{Creatinine production rate}}{\text{Creatinine clearance}} = \frac{\text{Production rate}}{\text{CL}_{\text{CR}}}$$

The only way that SCR could remain constant in the face of a decreasing CL_{CR} is if the production rate declines in parallel with clearance. In fact, this is quite likely on the basis that creatinine is an end product of muscle metabolism. Since muscle mass declines with age, it is reasonable to expect that the production rate of creatinine would decline.

The general conclusion is that renal function declines with age.

Hepatic Impairment: Xenobiotic Metabolism and Toxicity

Unfortunately, unlike the situation with renal impairment, there is no reliable quantitative endogenous measure of liver function that one can relate to drug metabolism. CL_H or ER_H and CL_{CR}, which is an excellent index of renal function, which quantitatively correlates with measures of drug excretion, such as CL_R, CL_S, or K. The search for an endogenous biochemical marker that would quantitatively relate to hepatic metabolic efficiency has been of long standing but, for the most part, unsuccessful. Currently, we are able to use the metabolism of certain probe markers or model compounds to assess the relative efficiency of a growing array of enzyme systems. For example, the results of metabolic tests using select probe compounds that undergo metabolism via specific isozymes, CYPs, of the cytochrome P450 system will permit placing subjects into phenotype groups of rapid or slow metabolizers (see Altman et al., 2013). The former is more an "all-or-none" assessment; there is no continuous measure of enzyme activity on a relative scale. The rapid developments in molecular biology and the area called "pharmacogenomics" hold out the promise that such an assessment could be achieved through DNA testing.

It is difficult to make generalizations concerning the influence of hepatic impairment on drug disposition for several reasons including the fact that there is no reliable quantitative index for measuring "hepatic function." With regard to hepatic disease states, there are acute vs. chronic hepatic disease conditions and these conditions vary according to the effect that they have on hepatic function. The term "hepatic impairment" may imply one cause or one effect, but in fact there are numerous factors involved. In terms of metabolic processes, there are a wide variety of isozymes with different intrinsic enzymatic activities and different cofactors and variables that influence activity. Each drug–isozyme pair will have a different set of enzymatic parameter values for metabolism (i.e., k_m and V_{MAX} or $CL_{u,i}$). It is not surprising, therefore, to find conflicting data making it difficult to reach unequivocal conclusions about liver disease and its effects on drug disposition.

Hepatic diseases will exert an effect on drug metabolism only when impairment results in measurable changes in endogenous biochemical factors (such as serum albumin levels). Table 17.3 lists a variety of biochemical parameters that change in response to liver disease and the resulting potential change in xenobiotic disposition.

Table 17.4 presents the most frequently used classification system for assessing the degree of liver impairment. Severity increases from Grades A to C from 1 point to 3 points per test (i.e., 5–6 points = mild impairment, 7–9 = moderate impairment, >9 = severe impairment). These scales are useful clinically for following the progression of the disease, but they offer little in terms of quantitating alterations in pharmacokinetic parameters.

Liver disease can influence absorption and drug disposition by altering the primary parameters: unbound intrinsic clearance, $CL_{u,i}$; unbound plasma fraction, f_u; and liver blood flow, QH. Alteration in those parameters can then produce measurable changes in several absorption and disposition parameters.

Altered Primary Parameter	Pharmacokinetic Parameter Effected
$CL_{u,i}$	$CL_s \rightarrow T_{1/2}$ (restrictive clearance)
	$ER_H \rightarrow F$ (nonrestrictive clearance)
f_u	$V \rightarrow T_{1/2}$
	$CL_s \rightarrow T_{1/2}$
Q_H	$CL_s \rightarrow T_{1/2}$ (nonrestrictive clearance)

Table 17.5 summarizes the pharmacokinetic changes for a number of (low) restrictively cleared compounds during liver disease (mostly cirrhosis). The healthy control values are listed

Table 17.3 Biochemical Changes and Potential Effects on Drug Disposition

Biochemical Measurement	Physiological/ Pathologic Alteration	Potential Pharmacokinetic Alteration	Problems in Interpretation
Prothrombin time	Acute ↓ protein synthesis	↓ metabolism	Low vitamin K
Serum albumin	Chronic ↓ protein synthesis	↓ metabolism, ↓ protein binding; ↑ Vd	Poor nutrition
Serum bilirubin			
Conjugated (direct)	Cholestasis	↓ biliary elimination of drugs	Prolonged elevations despite return of normal function
Unconjugated (indirect)	Hepatocyte dysfunction or ↓ extraction from blood	↓ metabolism	Hemolysis
Serum alkaline phosphatase	Cholestasis	↓ biliary elimination of drugs	↑ production
Serum aminotransferase [alanine (ALT)] [aspartate (AST)]	Hepatocyte damage	↓ metabolism	Normal in chronic disease. High elevations in acute disease that may not reflect hepatic malfunction

Source: Brouwer, K. L. R. et al., Influence of liver function on drug disposition, in *Applied Pharmacokinetics*, 3rd edn., W. E. Evans, J. J. Schentag, and W. J. Jusko, eds., Applied Therapeutics, Vancouver, WA, 1992, pp. 6-1–6-59.

Table 17.4 Classification Systems Used to Characterize Liver Impairment

Child–Turcotte Classification			
	Grade A	**Grade B**	**Grade C**
Bilirubin (mg/dL)	<2.0	2.0–3.0	>3.0
Albumin (g/dL)	>3.5	3.0–3.5	<3.0
Ascites	None	Easily controlled	Poorly controlled
Neurological disorder	None	Minimal	Advanced
Nutrition	Excellent	Good	Poor

Pugh's Modification of Child's Classification[a]			
	I Point	**2 Points**	**3 Points**
Encephalopathy (grade)	None	1 or 2	3 or 4
Ascites	Absent	Slight	Moderate
Bilirubin (mg/dL)	1–2	2–3	>3
Albumin (gnVdL)	>3.5	2.8–3.5	<2.8
Prothrombin time (sec > control)	1–4	4–10	>10

Source: Brouwer, K. L. R. et al., Influence of liver function on drug disposition, in *Applied Pharmacokinetics*, 3rd edn., W. E. Evans, J. J. Schentag, and W. J. Jusko, eds., Applied Therapeutics, Vancouver, WA, 1992, pp. 6-1–6-59.
[a] 5–6 total points = mild dysfunction, 7–9 = moderate dysfunction, >9 = severe dysfunction.

Table 17.5   Influence of Liver Disease on the Pharmacokinetics of (Low) Restrictively Cleared Drugs

Drug	Disease	Volume	$T_{1/2}$	Clearance
Ampicillin	C	59.1 ± 43.1 L[d]	1.90 ± 0.56 h[d]	280 ± 136 mL/min
	C	(19.5 ± 4.6) (V_{ss})	(1.31 ± 0.15)	(342 ± 80)
Chloramphenicol		49.9 ± 4 L[d]	10.45 ± 1.14 h[d]	59.2 ± 8.4 mL/min
		(65.9 ± 4) (V_{ss})	(4.6 ± 0.3)	(168.6 ± 9)
Chlordiazepoxide	C	428 ±108 mL/kg	40.1 ± 5.1 h[d]	7.6 ± 1.08 mL/kg/h
		(321 ± 77) (V)	(16.5 ± 3.6)	(13.8 ± 1.2)
	C	0.48 ± 0.14 L/kg	62.7 ± 27.3 h[d]	7.7 ± 2.1 mL/min
		(0.33 ± 0.06) (V_{ss})	(23.8 ± 11.6)	(15.4 ± 4.4)
Cimetidine	L[a]	1.4 ± 0.6 L/kg	2.9 ± 1.1 h	463 ± 145 mL/min
		(1.1 ± 0.4) (V)	(2.3 ± 0.7)	(511 ± 93)
Diazepam	C	1.74 ± 0.21 L/kg[d]	105.6 ± 15.2 h[d]	13.8 ± 2.4 mL/min
		(1.13 ± 0.28) (V)	(46.6 ± 14.2)	(26.6 ± 4.1)
Furosemide	C	533 mL/kg[d]	2.2 h	192 mL/min
		(210) (V)	(0.79)	(194)
	C	12 ± 3.5 L	129 ± 75 min	120 ± 36 mL/min
		(9.3 ± 3.7) (V_{ss})	(74 ± 18)	(142 ± 42)
Hexobarbital	C	1.14 ± 0.26 L/kg	509 ± 174 min[d]	1.88 ± 0.70 mL/min/kg
	(compensated)	(1–25 ± 0.24)(V.)	(340 ± 1 to)	(3.32 ± 0.99)
	C	1.57 ± 0.64 L/kg	1.017 ± 450 min[d]	1.26 ± 0.49 mL/min/kg
	(uncompensated)	(1.25 ± 0.24) (V)	(340 ± 110)	(3.32 ± 0.99)
Lorazepam	C	2.01 ± 0.82 L/kg	31.9 ± 9.6 h[d]	0.81 ± 0.48 mL/min/kg
		(1.28 ± 0.34) (V)	(22.1 ± 5.4)	(0.75 ± 0.23)
	AVH	1.52 ± 0.61 L/kg	25.0 ± 6.4 h	0.74 ± 0.34 mL/min/kg
		(1.2 ± 0.34) (V)	(22.1 ± 5.4)	(0.75 ± 0.23)
Oxazepam	C	60.9 ± 9.5 L	5.8 ± 1.1 h	155.5 ± 70.4 mL/min
		(61.2 ± 12.2) (V)	(5.6 ± 0.8)	(136.0 ± 46.3)
	AVH	51.7 ± 17.2 L	5.3 ± 0.7 h	137.4 ± 51.4 mL/min
		(47.7 ± 16.7) (V)	(5.1 ± 1.3)	(113.5 ± 30.7)
Prednisolone	CAH[b]	69 ± 13 L	3.0 ± 1.0 h	278 ± 79 mL/min
		(70 ± 8) (V)	(3.3 ± 1.0)	(256 ± 56)
Ranitidine	C	115 ± 32 L	166 ± 41 min	476 ± 139 mL/min
		(106 ± 35)	(124 ± 16)	(543 ± 126)
Theophylline	C	0.563 ± 0.08 L/kg	28.8 ± 14.3 h[d]	18.8 ± 11.3 mL/h/kg
		(0.482 ± 0.08) (V)	(6.0 ± 2.1)	(63.0 ± 28.5)
Tolbutamide	C	0.15 ± 0.03 L/kg	4.0 ± 0.9 h[d]	26 ± 5.4 mL/h/kg
		(0.15 ± 0.03) (V)	(5.9 ± 1.4)	(18 ± 2.8)
Warfarin	AVH	0.19 ± 0.04 L/kg	23 ± 5 h	6.1 ± 0.9 L/h
		(0.21 ± 0.02) (V)	(25 ± 3)	(6.1 ± 0.7)

Source: Williams, D. S., Drugs and the liver: Clinical applications, in *Pharmacokinetic Basis for Drug Treatment*, L. Z. Benet, N. Massoud, and J. G. Gambertoglio, eds., Raven Press, New York, 1984, pp. 63–76.

[a] L, type of liver disease not cited, enhanced CNS penetration of cimetidine.

[b] CAH, chronic active hepatitis.

in parentheses. In most cases, the value for CL_s decreases and $T_{1/2}$ increases (due to a decrease in $CL_{u,i}$). The latter may in part be due to an increase in apparent volume of distribution. In some cases, notably compounds undergoing phase II conjugation metabolism (e.g., lorazepam, oxazepam), there is no change in CL_s. In at least one case, there is an increase in CL_s, tolbutamide, which is probably a result of an increase in f_u.

Antipyrine is a frequently used marker for CYP 450 oxidative metabolism since it is completely metabolized, not plasma protein bound, and has a low clearance. The normal value of about 10 hours as a halflife for antipyrine increases markedly to over 25 hours for "all" liver disease states. Note, however, the extremely wide variation in the values (from about 10 to over 50 hours). One reason for this is the "lumping" together of all liver disease states into one category. The average values for $T_{1/2}$ depend upon the specific disease state but variation remains wide in all cases. Clearance would be a better parameter to compare. Variability remains very high in both the control and disease groups, and the raw data on the left-hand side indicate considerable overlap of the clearance values. If the clearance values are normalized for estimates of liver volume, as has been done on the right-hand side, both variability and overlap decrease considerably.

Reduced enzyme activity, as measured by $CL_{u,i}$, would be expected to decrease the hepatic clearance of low or restrictively cleared drugs, as noted earlier. In contrast, such changes should have no influence on the clearance of high or nonrestrictively cleared drugs. The clearance of such drugs will be influenced by changes in liver blood flow, Q_H, which is associated with many liver disease conditions. Table 17.6 summarizes the data for a number of high-clearance drugs. Clearance is reduced in most all cases and this is a reflection of altered blood flow.

Note also that bioavailability increases substantially in all cases. This is a reflection of a reduction in $CL_{u,i}$. A reduction in $CL_{u,i}$ results in a reduction in the hepatic extraction ratio, which in turn results in a decrease in the hepatic first-pass effect. In other words, the absolute oral bioavailability (F) increases. This will be most dramatic for drugs having a very high hepatic extraction ratio. Thus, if ER_H is 0.95 in normals and the value changes to 0.90 (less than a 5% decrease) in liver disease, the value for bioavailability (F) increases twofold, from 0.05 to 0.10. (Recall that $F = 1 - ER_H$.) An additional part of this increase in F is due to the existence of portal shunts that permit some of the absorbed dose to go directly from the gastrointestinal tract into systemic circulation. The propranolol and nicardipine concentrations following oral dosing in cirrhosis patients are much higher than those in normals.

The increased apparent volume noted for a number of compounds in Table 17.7 is a reflection of the altered (reduced) plasma protein binding of those compounds. The increase in the unbound fraction, f_u, is the result of decreased plasma protein concentrations (especially albumin) and/or the accumulation of endogenous compounds that would normally be eliminated by hepatic metabolism. The latter compounds may compete with drug for protein binding sites. Table 17.7 lists the percentage increase in the unbound fraction to plasma proteins for a variety of drugs. Binding either decreases or does not change, as noted in the table.

Hepatic metabolism and aging is very difficult to discuss as there do not appear to be any general rules. The most likely reason for this, unlike renal function, is that there are a host of factors that influence the efficiency of drug metabolism (e.g., genetics, nutrition, drugs, disease, live size, etc). There is huge variation among the population for hepatic clearance at any given age. It is not unusual to find a 10-fold range of clearances among otherwise normal, healthy subjects at a given age. On that basis, it is difficult, if not impossible, to tease out the effect of age per se on hepatic clearance (Rowland and Tozer, 2010).

The general impression is that hepatic drug clearance either decreases or remains unchanged with age at least for phase I metabolic processes. While not well proven, it appears that age has less of an effect on those compounds that undergo phase II metabolism (i.e., conjugation).

Table 17.6   Influence of Liver Disease on the Pharmacokinetics of (High) Nonrestrictively Cleared Drugs

Drug	Extraction Ratio	Disease	Bioavailability (% Change)	Volume[b]	$T_{1/2}$	Clearance
Chlormethiazole	0.9	C	+1000		8.7 ± 4.0 h	12.8 ± 4.8 mL/min/kg[d]
					(6.6 ± 2.4)[c]	(18.1 ± 29)
Labetalol	0.7	C	+91	526 ± 31 L[d]	170 ± 24 min	
					(805 ± 91) (V_{area})	(1.87 ± 26)
Lidocaine	0.7	C		2.22 ± 0.94 L/kg	343 ± 234 min[d]	5.2 ± 2.1 mL/min/kg[d]
				(1.70 ± 0.21) (V_{area})	(108 ± 70)	(9.2 ± 0.8)
		AVH[a]		310 ± 180 L/kg	160 min	13.0 ± 3.9 mL/min[d]
				(2.00 ± 0.5) (V_{ss})	(90)[e]	(20.0 ± 3.9)
Lorcainide		C			12.5 ± 4.5 h	814 ± 144 mL/min[d]
					(7.7 ± 2.0)	(1.002 ± 304)
Meperidine	0.5	C	+81	263 ± 28 L	359 ± 77 min[d]	523 ± 158 mL/min[d]
				(232 ± 53) (V_{ss})	(213 ± 25)	(900 ± 316)
		AVH		5.56 ± 1.8 L/kg	6.99 ± 2.74 h[d]	649 ± 228 mL/min[d]
				(5.94 ± 2.65) (V)	(3.37 ± 0.82)	(1.261 ± 527)
Metoprolol	015	C	+65	4.0 ± 0.3 L/kg	7.2 ± 1.2 h	0.61 ± 0.13 L/min
				(3.2 ± 0.2) (V)	(4.2 ± 1.1)	(0.01 ± 0.11)
Morphine	0.6–0.8	C		23 ± 1.3 L/kg	2.2 ± 1.3 h	1.153 ± 345 mL/min[d]
				(2.9 ± 2.4) (V_{ss})	(25 ± 1.5)	(1.233 ± 427)
Pentazocine	0.8	C	+278	356 ± 94 L	396 ± 115 min[d]	675 ± 296 mL/min
				(415 ± 107) (V_{area})	(230 ± 28)	(1.246 ± 236)
Propranolol	0.6	C	+42	380 ± 41 L[d]	11.2 ± 12 h	580 ± 140 mL/min
				(290 ± 17) (V_{area})	(4.0 ± 0.3)	(860 ± 90)
Verapamil	0.87	C		481 ± 141 L[d]	815 ± 516 min[d]	0.545 ± 0.181 L/min[d]
				(296 ± 67) (V_{ss})	(170 ± 72)	(1.571 ± 0.405)
Isoretinine		C	+140[f]	9.17 L/kg	840 min	1.22 L/min
				(6.15) (V_{ss})	(220)	(1.26)

Source: Williams, D. S., Drugs and the liver: Clinical applications, in *Pharmacokinetic Basis for Drug Treatment*, L. Z. Benet, N. Massoud, and J. G. Gambertoglio, eds., Raven Press, New York, 1984, pp. 63–76.

[a] C, cirrhosis; AVH, acute viral hepatitis.
[b] V_{ss}, volume of distribution at steady state; V, volume of distribution (one compartment); V_{area} clearance × 0.693/$T_{1/2}$.
[c] Numbers in parentheses indicate values observed in healthy controls.
[d] Statistically significant differences between patients and healthy controls.
[e] Mean values only reported.
[f] Because of a sixfold variability, caution is suggested in interpretation of this change in bioavailability (52).

Table 17.7   Increase in the Unbound Fraction to Plasma Proteins in Liver Disease

Drug	Diseases	Percentage Increase in Fraction Unbound
Highly extracted drugs		
Udocaine	AVH	No change
Meperidine	AVH	No change
Morphine	AVH/C	15
Propranolol	AVH/C	38
Poorly extracted drugs		
Amobarbital	AVH/C	38
Azapropazone	CAH/C	477
Diazepam	C	210
Diazepam	C	65
Phenylbutazone	C	400
Phenylbutazone	AVH/C	500
Phenytoin	AVH	33
Phenytoin	C	40
Quinidine	C	300
Tolbutamide	AVH	28

Source: Williams, D. S., Drugs and the liver: Clinical applications, in *Pharmacokinetic Basis for Drug Treatment*, L. Z. Benet, N. Massoud, and J. G. Gambertoglio, eds., Raven Press, New York, 1984, pp. 63–76.
Note: AVH, acute viral hepatitis; C, cirrhosis; CAH, chronic active hepatitis.

Impaired GI Absorption

Gastrointestinal absorption has been claimed for many years to be impaired in the elderly. There is simply no good evidence for this and, in fact, the former statement has been made because of poor experimental design and incorrect interpretation of data. There are definitely many changes in the gut that could affect absorption efficiency. Some of these are noted in Table 17.8.

There is no very good information about a number of the factors listed in Table 17.7. There is a greater incidence of achlorhydria (lack of acid secretion in the stomach), which may have some implications in terms of drug dissolution and drug stability, but at the present time, this condition does not appear to be an important clinical issue. Gastric emptying rate and intestinal transit rate are expected to be reduced with age; there is the general thought that gut activity slows with aging. This is difficult to conclude, however, and the data shown in Table 17.9, which are the only data

Table 17.8   Factors That Are Known to or Thought to Change with Aging That May Affect Gastrointestinal Absorption

GI fluid pH
GI fluid contents
Gastric emptying rate
Intestinal transit rate
GI blood flow
GI surface area and "membrane" characteristics
Nutritional intake and eating habits
Age-related drug ingestion altering physiology
or affecting absorption of other drugs
Age-related GI disease

Table 17.9   Two Studies That Have Examined the Influence of Age on Gastric Emptying Half-Time (Inversely Related to Emptying Rate)

	Age, Years		Liquid Meal		
n[a]	Mean	Range	Mean	Range	
	26	23–31	50	21–132	
	77	72–86	123	67–4541	P < 0.001

	Age, Years		Liquid Phase		Solid Phase	
n	Mean	Range	Mean	SEM[b]	Mean	SEM
10	31	24–51	68	7 ns[c]	104	10 ns
10	76	71–88	94	13 ns[c]	105	17 ns

Note: The top study indicates no difference.
[a] Number of subjects.
[b] Standard error of the mean.
[c] Not significantly different.

available, are directly conflicting. In one case, there is no change and in the other, the elderly empty more slowly than younger subjects. Even less is known about intestinal transit rates, but once again, the prevailing thought is that it decreases with age.

One of the most interesting stories concerns the use of d-xylose for the estimation of GI absorption. That carbohydrate is often used to assess the presence of malabsorption syndromes, especially in the case of sprue. It has also been applied to the elderly and pediatric populations. Unfortunately, the data have been totally misinterpreted and this has led to the general statement about impaired absorption with aging. The basis of the method is quite simple; ingest a 5 or 25 g dose of d-xylose and collect urine for 5 hours. The amount excreted is then compared to a normal range. In reviewing the literature, one can plot, from a number of different studies, the percentage dose recovered in 5 hours as a function of age. Note the lines decrease, which suggests impaired absorption with age. However, it is very difficult to explain line A that is obtained following an intravenous dose of d-xylose; it too declines with age! The only explanation for this is not a decrease in absorption (after all, absorption is complete, 100%, after intravenous dosing) but a reduction in elimination. The latter makes sense, when we realize that, as will be discussed later, renal function declines with age and that we are therefore obtaining an incomplete urine collection.

The general conclusions with regard to gastrointestinal absorption are that the rate of absorption may decline with age but there are no differences in the extent of absorption as a function of age. The only exception to the latter rule is drugs that have a high hepatic extraction ratio and that undergo substantial first-pass metabolism. For such drugs, absorption may increase with age as a result of reduced hepatic clearance (Brus and Herman, 1971).

There are virtually no data about the influence of age on drug absorption by other routes of administration such as intramuscular, transdermal, pulmonary, etc.

Distribution may be altered as a result of changes in plasma protein concentration, as a result of changes in anatomy and due to blood flow differences. Changes in plasma protein binding may result from the preceding observation, but it may also occur due to the use of many drugs by the elderly and the consequent interaction in displacing one compound by one or more other drugs.

Another major change that occurs with aging is with regard to the relative body content of water and adipose tissue. In both males and females as regards the relative percentage of weight, adipose tissue increases with age, while water content (or lean body mass) declines with age.

Another important issue in drug distribution is with regard to blood flow. Age per se does not appear to alter cardiac output. However, among the population in which there is substantial coronary artery disease, there appears to be an inverse relationship with age. One needs to first define

the question in order to provide a correct answer. The following are the conclusions with regard to drug distribution and aging:

- Plasma protein binding either decreases or does not change with age.
- Clearance may change (increase) as a result of altered binding (low-clearance drugs).
- Apparent volume of distribution will increase for lipid-soluble drugs and decrease for water-soluble drugs.
- Half-life may change as a result of a change in apparent volume.
- Blood flow decreases in most elderly subjects and this may alter the clearance of nonrestrictively cleared drugs.

Pregnancy

There are numerous and substantial changes that occur during pregnancy, and while there is not a great deal of quantitative information with regard to drug disposition and response, several of these factors can be discussed. There is little quantitative information with regard to the effect of pregnancy on the efficiency of the gastrointestinal absorption process or, for that matter, absorption by other routes of administration. Several changes in the gastrointestinal tract do occur, however, which include a reduction in gastric acid secretion, an increase in mucous secretion, and a slowing in gastric emptying and intestinal motility. Any of these changes may affect the drug dissolution and absorption processes, at least in theory, but as yet we do not have the data to indicate the existence of such alterations in absorption. Increased peripheral blood flow might also suggest more rapid absorption following intramuscular or subcutaneous and, perhaps, transdermal dosing.

There are considerable anatomical changes that occur during pregnancy and that may affect drug distribution. This increase in weight in terms of fluid volumes, blood, and fat is illustrated in Table 17.10.

The increased volumes of both water and fat will likely increase the apparent distribution space for water-soluble and lipid-soluble drugs. An additional consideration, however, is the distribution space afforded by the fetus (assuming the drug can traverse the placenta) and breast milk (assuming the drug can undergo mammillary transfer).

The other major factor that may affect distribution volume as well as clearance (and, therefore, half-life) is plasma protein binding. As with other factors, one must consider the time course of any possible change over the normal duration of pregnancy. Serum albumin concentration declines with age, and for many of the drugs studied to date, plasma protein binding also decreases, which results in a greater fraction of unbound drug.

There are substantial changes in hemodynamic functions such as cardiac output and blood flow to different body regions. These changes are shown in Table 17.11.

The implications of the aforementioned changes in terms of drug distribution have not been clearly delineated, but the findings suggest that there would be an increase in distribution volume during pregnancy, which then returns to normal some time following birth of the child.

Table 17.10   Change in Blood Distribution during Pregnancy

Blood Changes in Pregnancy			
Parameter	Late Pregnancy	Nonpregnant State	Increase (%)
Blood volume (mL)	4820	3250	48
RBC volume (cells/mm³)	1790	1355	32
Hematocrit (%)	37.0	41.7	

Source: Data from Pritchard, J. A., *Anesthesiology*, 26, 393, 1965.
Note: RBC, red blood cell.

Table 17.11 Hemodynamic Parameters throughout Pregnancy

		Patient			
	Position	1st Trimester	2nd Trimester	3rd Trimester	Postpartum
Heart rate (beats/min)	L	77 ± 2	85 ± 2	88 ± 2	69 ± 2
	5	76 ± 2	84 ± 2	92 ± 2	70 ± 2
Stroke volume (mL/min)	L	75 ± 3	86 ± 4	97 ± 5	79 ± 3
	S	82 ± 5	85 ± 4	87 ± 5	79 ± 3
Cardiac output 1/mm/m²	L	3.53 ± 0.21	4.32 ± 0.22	4.85 ± 0 27	3.30 ± 0.17
	S	3.76 ± 0.24	4,19 ± 0.21	4.54 ± 0.28	3.33 ± 0 21
Left ventricular election time (ms)	L	302 ± 2	290 ± 5	281 ± 4	310 ± 5
	S	301 ± 3	286 ± 4	260 ± 4	307 ± 5
Systolic blood pressure (mm Hg)	L	98 ± 2	91 ± 2	95 ± 2	97 ± 2
	S	106 ± 2	102 ± 2	106 ± 2	110 ± 2
Diastolic blood pressure (mm Hg)	L	53 ± 2	49 ± 2	50 ± 2	57 ± 2
	S	57 ± 2	60 ± 1	65 ± 2	65 ± 1

Note: L, lateral; S, supine.

Table 17.12 Changes in Kidney Function in Pregnancy

Time	Renal Plasma Flow (mL/min)	Glomerular Filtration Rate (mL/min)
13.0-week pregnancy	804.67	161.33
20.8-week pregnancy	749.13	157.11
38.0-week pregnancy	589.00	146.00
20-week postpartum	491.00	100.00
80-week postpartum	549.00	97.00

There is a very dramatic increase in renal function as judged by estimates of glomerular filtration (e.g., creatinine and inulin clearances). This is illustrated in Table 17.12.

This increase in renal function has its direct counterpart in the renal clearance of drugs that undergo excretion by the kidney. Ampicillin, for example, is primarily excreted unchanged by the kidney and has a greater clearance in the same women during pregnancy (613 mL/min) compared to the value after giving birth (394 mL/min). Elimination half-life is also shorter and the apparent volume of distribution is larger.

Findings similar to the aforementioned have been made for other water-soluble antibiotics (e.g., cephalosporins) and digoxin, among other renally excreted drugs. Therefore, it is very likely that larger than usual doses of such drugs will need to be given to pregnant women in order to achieve the desired steady-state plasma concentration and response.

As noted under the discussion of age, it is far more difficult to address the issue of drug metabolism because of the large number of variables that affect hepatic metabolism, and once again, there are only a limited number of studies available. Two studies examined the difference in disposition of the drug, metoprolol, in the same women during and after pregnancy. These results are summarized in Table 17.13.

These data suggest that there is an increase in hepatic metabolic activity during pregnancy, at least for the enzyme system responsible for metoprolol metabolism. General conclusions concerning the metabolism of other drugs during pregnancy are far from being unequivocal. Metabolic clearance is affected by several factors, some of which have been noted earlier. First, intrinsic hepatic or metabolic clearance may change in response to, for example, hormonal alterations during pregnancy. In fact, it appears that many, if not all, of the changes in metabolic efficiency are related to hormonal activity, as will be noted later when we consider the influence of oral contraceptives

Table 17.13   Metoprolol Absorption and Disposition Parameters in Women during and following Pregnancy

Parameter	Pregnant	Nonpregnant
Oral dose (100 mg)		
CL_o (mL/min/kg)	362	82
$T_{1/2}$ (h)	1.27	1.70
IV dose (10 mg)		
CL_s (L/min)	1.38	0.65
V (L/kg)	6.87	3.85
$T_{1/2}$ (h)	5.38	5.36
Oral dose (100 mg)		
CL_o (L/min)	9.56	1.71
F	0.21	0.42

Note: Studies done in 5 women during 3rd trimester of pregnancy and 3–6 months after giving birth.

on drug metabolism. Furthermore, the effects of hormones on drug metabolism are not always clear-cut but often appear contradictory. The latter point probably reflects the fact that there are other factors, currently not well recognized or understood, that have an impact on the overall metabolic disposition of drugs. For example, an increase in testosterone concentrations increases hepatic microsomal enzyme activity, thereby enhancing the rate of hepatic metabolism of some drugs. In contrast, progesterone and estradiol may act as inhibitors of certain enzymatic processes and thus reduce the rate of metabolism.

The other factors that play a role in hepatic clearance are plasma protein binding and hepatic blood flow. The former has been discussed earlier, and assuming a general decrease in binding during pregnancy, one would expect an increase in hepatic clearance. Liver blood flow, on the other hand, does not appear to be dramatically affected during pregnancy and, therefore, should have a minimal effect on drug metabolism.

The preceding issues have important ramifications in terms of appropriate drug dosing during pregnancy in order to maintain the desired therapeutic response and minimize adverse, toxic effects. One can make a good argument for therapeutic drug monitoring for those drugs with a narrow therapeutic range along with careful, continued monitoring of the response to the drug. Making the aforementioned issues more complicated is a concern for the fetus that is exposed to the drugs that the mother is taking. To minimize adverse effects on the fetus, it may be necessary to select a drug that undergoes minimal placental transfer and/or exerts little effect on the fetus. An additional complication arising after birth is the mammillary transfer of drug and subsequent exposure of the breastfeeding infant.

We have long known that there are certain cycles or rhythms that biological systems undergo that may have a dramatic affect on various aspects of the system. The term given in this area of study is "chronobiology" and it has received considerable interest in recent years. The menstrual cycle is a good example of chronobiology, yet its implications are poorly understood with regard to drug disposition and pharmacologic response. It has been only in very recent years that we have begun to learn of the impact that menstruation may have on the outcome of therapy.

It is not clear whether or not gastrointestinal absorption is altered during the menstrual cycle as this process has not been thoroughly studied. One well-designed study that examined the oral absorption of d-xylose indicates no significant differences during the follicular (days 4 and 5), ovulatory (days 16 and 17), or luteal phases (days 23 and 24).

A 20% increase in creatinine clearance has been reported between the beginning and end of the menstrual cycle, which is consistent with the approximately 24% increase in d-xylose renal clearance. In contrast to these findings, however, no changes were noted in creatinine clearance and tobramycin pharmacokinetics during the corresponding phases of the menstrual cycle in another study.

Table 17.14 Pharmacokinetic Parameters of Methaqualone during the Menstrual Cycle

Parameter	Day of Cycle	
	Day 1	Day 15
Half-life (h)	16.3	11.6
Clearance (mL/min/kg)	1.72	3.20
Volume (L/kg)	2.12	2.84

As a result, and because there is a lack of other basic studies, one cannot reach a general conclusion concerning the changes in renal function during the menstrual cycle.

Unfortunately, but not surprisingly, similar disparities exist with regard to consideration of drug metabolism during the menstrual cycle. To date, there have been at least two different studies that have examined the pharmacokinetics of the model compound, antipyrine, during different times of the menstrual cycle. The results of both of these studies suggest that there is no time-dependent alteration in antipyrine metabolism during the menstrual cycle.

One positive finding is the results of a study that examined methaqualone pharmacokinetics during days 1 and 15 of the cycle. There is a very dramatic difference in the values for clearance and half-life, and although the mechanism(s) for this alteration is not currently known, the investigators suggest a hormonal action (Table 17.14).

Influence of Gender on Xenobiotic Absorption, Disposition, and Toxicity

The effect of gender on animal and human responses to toxicants has long been passively recognized but not acted on (Palotta et al., 1962; Shanor et al., 1961). It has become quite clear in recent years that women's health and health issues have not been adequately studied. As a result, we understand far less about the diagnosis of illness and its treatment in women than in men. For the same diagnosis, women are less likely than men to receive important diagnostic or therapeutic modalities (e.g., renal transplantation, cardiac catheterization). The issue, however, is not so straightforward and is complicated by several factors such as age associated with heart disease (at an earlier age in men than in women) and the dangers of certain therapeutic maneuvers (e.g., catheterization) at those ages. It is important to recognize that the medical treatment for women is based upon a "male" model. "The results of medical research on men are generalized to women without sufficient evidence of applicability to women." This statement is best supported by consideration of the large-scale clinical research studies that have been conducted in this country over the past several decades:

- The Physicians Health Study examined the use of aspirin for coronary artery disease (22,071 male, 0 female).
- The Multiple Risk Factor Intervention Trial examined the modification of risk factors to prevent heart disease (15,000 male, 0 female).
- The Veterans Administration Cooperative Study showed the benefits of coronary surgery in angina patients.
- The Baltimore Longitudinal Study of Aging, which has been ongoing since 1958, began to include women only since 1978.

Consider the following facts:

- Women will constitute the larger population and will be the most susceptible to disease in the future.
- Overall, women have worse health than men.
- Certain health problems are more prevalent in women than in men.
- Certain health problems are unique to women or affect women differently than they do in men.

Life Expectancy, 1989		
	Men	**Women**
Total population	71.8	78.6
White	72.7	79.2
Black	64.8	73.5
Hispanic	69.6	77.1
Native American	*	*
Asian Pacific Islanders	*	*

* No information.

Percentage of Women within the Aging Population		
Year	**65+**	**85+**
1900	49.5%	55.6%
1980	59.7	69.6
1990	59.7	72.0
2020	60.0	73.0

The Assistant Secretary for Health established a Public Health Task Force on Women's Health Issues in 1983. One result was the publication of "Women's Health: Report of the Public Health Service Task Force on Women's Health Issues" (vol. 1, 1985; vol. 2, 1987). One of the most important recommendations of this task force was the recommendation that "biomedical and behavioral research should be expanded to ensure emphasis on conditions and diseases unique to, or more prevalent in, women in all age groups."

A consequence of these activities was the creation of the Office of Research on Women's Health (ORWH) within the Office of the Director of the National Institutes of Health (NIH). Currently, consideration is being given to establishing a permanent office for the ORWH within the NIH. The objectives of the ORWH are the following:

- Any research supported by NIH must adequately address issues related to women's health.
- Women must be appropriately represented in any clinical research, especially clinical trials.
- Foster an increase in the enrollment of women in biomedical research, especially in decision-making roles within clinical medicine and the research environment.

A public hearing in June 1991 was held for the purpose of determining the major needs for research into women's health, and the testimony given indicated that the following issues should receive attention (among other issues):

- Cancer prevention (especially breast cancer)
- Cardiovascular disease
- Osteoporosis
- Autoimmune diseases affecting women
- Hormonal cycles and how these may affect "absorption, disposition, action, and elimination of drugs"
- Sexually transmitted diseases
- Work site safety

- Domestic violence
- AIDS
- Pre- and postnatal care

The ORWH has begun the largest clinical project of its kind ever undertaken in the United States: "The Women's Health Initiative." The purposes of this project are to

- Decrease prevalence of cardiovascular disease, cancer (especially breast cancer), and osteoporosis
- Develop recommendations on diet, hormone replacement therapy, diet supplements, and exercise
- Evaluate effectiveness of various strategies for motivating older women to adopt health-enhancing behaviors

The project is expected to involve 150,000 women at 45 centers for up to 14 years and cost about $625 million. The health issues to be addressed will be divided according to age and processes:

- Consideration of age
  - Birth to young adulthood (birth to 15 years)
  - Young adulthood to perimenopausal years (15–44 years)
  - Perimenopausal to mature years (45–64 years)
  - Mature years (65+ years)
- Consideration of process
  - Reproductive biology
  - Early developmental biology
  - Aging processes
  - Cardiovascular function and diseases
  - Malignancy
  - Immune function and infectious diseases

Leading Causes of Death in Women*

Condition	Number
Heart disease	379,754
Cancer	226,960
Cerebrovascular disease	90,758
Pneumonia/influenza	40,828
Chronic obstructive Pulmonary disease	331,914
Accidents	31,279
Diabetes	23,393
Atherosclerosis	13,759
Septicemia	11,793
Nephritis	11,512

* National Center for Health Statistics, 1988.

Certain conditions are more prevalent among women than in men and these include the following:
   Cardiovascular disease:

- Stroke accounts for a greater percentage of deaths among women than in men at all stages of life.
- Half of all women, but only 31% of men, who have heart attacks die within 1 year.
- About 90% of all heart disease deaths among women occur after menopause.
- One in 9 women 45–64 years have some clinical cardiovascular disease, increasing to 1 in 3 at age 65 and older.

Mental disorders:

- The rate of affective disorders (depression, etc., ~7% in women) is about twice the rate in men.
- In elderly women depression affects about 3.6% vs. 1% in men.

Alzheimer's disease:

- It has higher incidence among women.

Osteoporosis:

- It affects over 24 million Americans, primarily women.

Incidence of Osteoporosis in Women	
Age Group	% Incidence
45–49	17.9%
50–54	39.2
55–59	57.0
60–64	65.6
65–69	73.5
75+	89.0

- Hip fractures are the most serious consequence of osteoporosis (ca. 250,000/year).
- Causes 1.3 million bone fractures/year.
- Five hundred thousand vertebral fractures occur per year and about one-third of women over age.
- Sixty-five women will suffer at least one vertebral fracture.

Sexually transmitted disease:

- Six million women per year are affected (one-half are teenagers).
- Fifteen to 20 million women have chronic genital herpes or human papillomavirus.
- Women are the fastest-growing population with AIDS.

Immunologic diseases:

- Autoimmune thyroid diseases have a 15:1 ratio of women to men.
- Rheumatoid arthritis has a 3:1 ratio of women to men.
- Systemic lupus erythematosus occurs nine times more often in women than men.
- Systemic sclerosis affects women four times as often as men.
- Diabetes mellitus and multiple sclerosis occur more often in women.

Certain health problems are unique to women or affect women differently than they do in men:

- Cancer is the second leading cause of death; it is the leading cause of premature death in women.
- Lung cancer has exceeded breast cancer as the leading cause of death due to cancer (51,000 vs. 45,000 in 1991).
- One in 9 women will develop breast cancer vs. 1 in 20 in the 1960s.
- Two and one-half million women acquire chlamydial genital infections annually.
- One million women are treated for pelvic inflammatory disease annually.
- Incidence of involuntary infertility and ectopic pregnancies has quadrupled in the past decade due to sexually transmitted diseases.

- Due to perinatal transmission, AIDS is the leading cause of death among Hispanic children and the second leading cause of death among Black children.

The FDA restricts the inclusion of women of childbearing age in early clinical trials except for studies involved with life-threatening diseases. These restrictions are currently being reconsidered:

- Gender-related differences in drug disposition in nonhuman mammals, especially in metabolism, have been known for some time. There are also several impressive differences in the magnitude of response to selected drugs in male and female rats (e.g., anesthetics and barbiturates). The prevailing thought up to just a few years ago was that these differences did not apply to humans.

The gender-related differences in drug metabolism in animals have been shown to depend upon the specific drug, metabolizing enzyme system, age, and animal species, among other variables. The differences in drug metabolism between the genders appear to be as a result of hormonal differences between the sexes (Kato and Gillette, 1965).

The lack of unequivocal information concerning differences in drug disposition between males and females has been the result of limited studies, small number of subjects in each study, and the influence of confounding variables. With regard to confounding variables, there is often no control for or consideration given to factors such as differences in age, smoking status, use of other drugs (especially oral contraceptives, caffeine, and ethanol), time during the menstrual cycle, and diet. The latter factors may exert a profound influence on the results of any study attempting to examine and compare the disposition of a drug as a function of gender. Several reviews on this topic have appeared in the literature.

Few, if any, studies have indicated substantial gender-related differences in the rate or extent of drug absorption, which might prove to be clinically important. The detection of such differences requires intensive sampling that is seldom applied in the typical clinical study. Furthermore, the incorrect analysis of data may lead to an erroneous conclusion (e.g., comparisons of blood concentrations only that do not take into account differences in drug clearance).

There is one important and interesting exception to this general statement that may have a counterpart for certain other drugs. Women have greater blood ethanol concentrations compared to men following oral doses of ethanol, and this has been ascribed to differences in body build (i.e., smaller % body weight, which is fat-free in women and into which ethanol distributes). Blood concentrations are similar, however, following intravenous dosing. Ethanol is metabolized in the stomach by the enzyme, alcohol dehydrogenase, before it is absorbed into the systemic blood circulation. The latter is an example of gastrointestinal or gastric "first-pass" metabolism. It appears that women have a much lower gastric alcohol dehydrogenase enzyme activity than men, which results in less metabolism in the stomach, and consequently, a greater part of the ethanol dose is absorbed into the blood stream. Women, therefore, will have a greater blood ethanol concentration than men at comparable oral doses. The approximate absolute oral bioavailability of ethanol in women was 91% compared to 61% for men.

There are examples of other drugs that undergo some form of gastrointestinal metabolism prior to absorption, which may be prone to the same effect noted for ethanol. For example, L-DOPA is inactivated in the stomach by a decarboxylase enzyme. Does the activity of this enzyme vary between the genders?

Another potential gender-related difference in oral bioavailability could involve hepatic first-pass metabolism. The concept is identical to that noted earlier for gastric metabolism, the only difference being the site of metabolism. What determines the significance of the hepatic first-pass effect is the hepatic clearance value of the drug; the greater the clearance, the greater the first-pass effect and the lower the systemic oral bioavailability. Therefore, if there are gender-related differences in the hepatic clearance of high-clearance drugs, one would expect there to be a corresponding

difference in gastrointestinal absorption or bioavailability. While there is currently little information upon which to make a conclusion, there are suggestions that hepatic clearance of certain drugs differs between the genders (discussed later in the section on metabolism).

Drug distribution throughout the body depends upon several factors including plasma protein and tissue binding and body build with regard to relative amounts of adipose and fat-free tissues. These factors, in turn, determine the apparent volume of distribution of a drug that affects the elimination half-life (i.e., half-life increases as the apparent volume of distribution increases, assuming that elimination clearance of the drug remains the same). Plasma protein binding may also affect the clearance of a drug depending upon the nature of the clearing process.

The current literature with regard to plasma protein binding differences between the genders is conflicting and inconclusive. Some studies suggest a lower binding in women compared to men for certain highly bound drugs, and other studies suggest no differences for the same drugs. The reasons for this disparity include the inclusion of few subjects and the lack of control for a number of variables that may alter binding (e.g., plasma protein concentration, age, health and nutritional status, other drugs, smoking, etc). As a result, no definitive conclusion can be reached but the general impression is that differences, if they exist, are not dramatic.

There are substantial differences between the genders, however, with regard to body build, which are further magnified with age (as discussed in the section on age). The major difference here is the fact that women have a greater percentage of body weight that is adipose tissue compared to men; conversely, women have a smaller percentage of body weight that is fat-free or that contains water. Therefore, a lipid-soluble drug (e.g., thiopental) will have a larger apparent volume of distribution (i.e., it will occupy a larger space) in women compared to men of the same age on a body weight basis (e.g., volume/kg). In contrast, a water-soluble drug (e.g., ethanol) will have a smaller volume of distribution in women compared to men of the same age. These differences can become important when administering a loading dose of a drug and in terms of the elimination half-life.

This is the most difficult topic to discuss rigorously since there is virtually no consensus about how gender affects drug metabolism in general, although specific drug examples may be discussed. The reason for this dilemma is the fact that there have been far too few adequate research studies that have employed a sufficient number of subjects and the fact that there are a host of variables, in addition to gender, which may affect drug metabolism. A brief listing of those factors would include consideration of genetics, age, health status, nutritional status, smoking status, and the use of other drugs (including ethanol, caffeine, and drugs of abuse). Furthermore, when considering gender per se, one must also control the time of the menstrual cycle and the possibility of pregnancy in addition to the use of oral contraceptives. Clearly, it is very difficult to factor-out gender differences as being responsible for any observed differences in metabolism when there are so many other variables. There must be exquisite care given to the control of all aspects of such a comparative study in order to obtain statistically valid conclusions.

Furthermore, it would be far more instructive to obtain estimates of metabolic (or hepatic) clearance of a drug compared to elimination half-life, since the former is an adequate measure of the inherent ability of the liver to metabolize drug, while the latter reflects clearance as well as volume (as noted earlier). Unfortunately, not all investigations recognize this difference and they report half-life more often than clearance.

The relationship among these three parameters is presented in the following equation:

$$T_{1/2} = \frac{0.693 \times \text{Volume}}{\text{Clearance}}$$

Differences in clearance as a function of gender must also consider the possibility of differences in plasma protein binding that may affect the clearance of certain drugs (those with low-clearance

values). A further complication in comparisons of clearance values is whether or not the values are adjusted for body weight, since this normalized value is usually greater in women by virtue of their weighing less than men.

At this time, it is almost counterproductive to list the results of the several studies that have attempted to compare metabolic drug efficiency in men and women, since the results of one study are often diametrically opposite the findings from another.

This is the case, for example, for many benzodiazepine derivatives that undergo oxidative (phase I) biotransformation (e.g., diazepam, chlordiazepoxide, etc.) and the frequently used model or test compound, antipyrine.

One recent study examined the role of gender in propranolol kinetics subsequent to an earlier report that suggested that females have higher concentrations of the drug compared to males after long-term oral dosing following a myocardial infarct. The investigators report that the oral clearance of propranolol is greater in males (leading to a greater first-pass effect, as discussed in the section on absorption). Females, therefore, have greater plasma concentrations of the drug compared to males, supporting the preliminary observation noted earlier. Complicating this finding, however, is the fact that while metabolic clearance was greater for two pathways (a side-chain oxidation and glucuronidation), it was not different for at least one other pathway (ring hydroxylation).

To further complicate this issue is the recently discovered gender- and age-specific enantiomer-selective metabolic differences. The considerable recent interest in drug enantiomers (on the basis of different pharmacologic and pharmacokinetic behaviors) has led to a greater ability to assay these different stereoisomers in biological fluid with the resulting greater understanding of what factors influence the disposition of those forms. One recent study, for example, indicates that there is an age-dependent gender effect as well as a gender-dependent age effect in the metabolism of the R-enantiomer of mephobarbital. In contrast, little difference exists in the metabolism of the S-enantiomer. While we have been aware of the age dependence in the metabolism of enantiomers (e.g., hexobarbital), this is the first example of a gender dependence. Undoubtedly, other examples will be forthcoming.

In contrast to the difficulty in reaching any unequivocal conclusions about gender-related differences in phase I metabolic processes, one investigator concludes that there is a more consistent gender-related trend in the metabolic clearance of those benzodiazepine derivatives that undergo conjugation reactions (phase II). A comparison among several of those drugs is shown in Table 17.15.

These data, however, remain far too limited to allow us to reach any valid general conclusions concerning consistent differences between the sexes for the metabolism of those drugs that undergo phase II metabolism. For that reason, recommendations cannot be made with regard to the need to alter dosing regimens as a function of gender. It is quite clear that there is a need for additional studies to clarify the influence of gender on drug metabolism. As alluded to the aforementioned and as will be noted later, the effect of gender is further complicated by consideration of age.

In addition to metabolism, urinary excretion is another major route of drug elimination from the body. Unlike metabolic processes, there are fewer variables that affect renal excretion of

Table 17.15 Clearance of Selected Benzodiazepine Derivatives Undergoing Phase II Conjugation Reactions in Normal, Young Males and Females

Drug	Clearance (mL/min)	
	Male	**Female**
Lorazepam	77	55
Oxazepam	88	50[a]
Temazepam	97	68[a]

[a] Significantly different from male value.

drugs (e.g., urine flow, urine pH). As a result, the renal clearance of a drug is reasonably consistent among people with similar kidney function. Renal clearance is often estimated by measurement of creatinine clearance or, more often, by serum creatinine concentration. The latter, however, can be quite misleading since factors such as age and body build affect the relationship between concentration and clearance. The following useful relationship between creatinine clearance (CLCR) and serum creatinine concentration (SCR) has been determined from many male subjects:

$$\mathrm{CL_{CR}}\left(\mathrm{mL/min}\right)_{\mathrm{MALE}} = \frac{\left(\text{Ideal body weight,kg}\right)\left(144 - \text{age,year}\right)}{72 \times \mathrm{SCR}\left(\mathrm{mg\%}\right)}$$

The corresponding equation for "females" requires multiplication by a factor of 0.86. The latter correction is a result of the fact that creatinine is an end product of muscle metabolism and females have a smaller muscle mass compared to males. This relationship is rewritten as follows. Therefore, at equal body weight, age, and serum creatinine concentration, the male will have a greater creatinine clearance and, presumably, more efficient kidney function and a greater ability to excrete drugs. To the best of this writer's knowledge, surprisingly, there has not been a systematic investigation of gender-related differences in renal function and renal excretion of drugs.

$$\mathrm{CL_{CR}}\left(\mathrm{mL/min}\right)_{\mathrm{FEMALE}} = \frac{0.86\left(\text{Ideal body weight,kg}\right)\left(144 - \text{age,year}\right)}{72 \times \mathrm{SCR}\left(\mathrm{mg\%}\right)}$$

Figure 17.1 is a plot of the percentage function remaining for several body functions vs. age. Note that this is a linear scale indicating that the decline in function occurs at a constant rate. (Also note that there is no precipitous drop at age 65 years!)

The reasons for considering the elderly as a special segment of the population and some of the complications in conducting gerontologic research are outlined in Table 17.16.
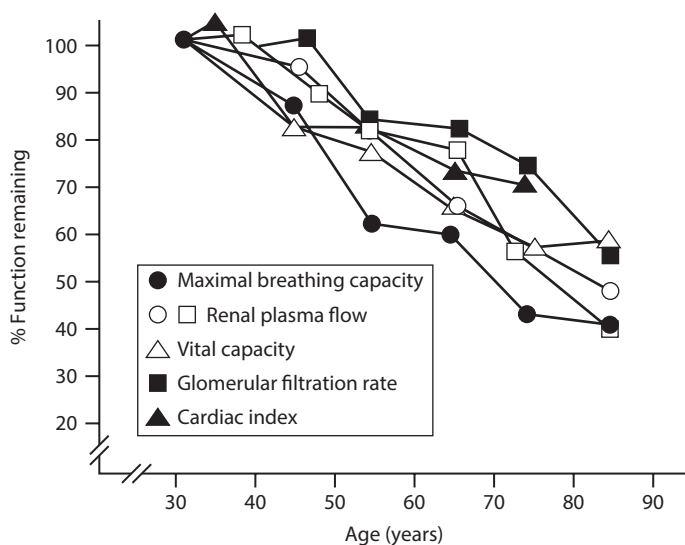
Figure 17.1   Plot of the percentage function remaining for a variety of body functions versus age.

Table 17.16   Characteristics of the Elderly That Warrant Their Being Considered as a Special Segment of the General Population

Consideration	Characteristics
Population	The elderly (i.e., those older than age 65) currently represent about 12% of the U.S. population; this percentage is expected to increase to about 15% by year 2000.
Health	The elderly experience a greater incidence of disease, physical impairments, and physiological disorders than do younger adults.
Institutionalization	The elderly occupy a greater share of hospital beds ($\approx$33%) and long-term care facilities than do younger adults.
Drug use	The elderly consume more drugs ($\approx$25% of total use) per capita than do younger adults.
Drug effects	The elderly experience a greater incidence of adverse drug effects and drug interactions than do younger adults.

Factor	Complication
Age, definition	Chronological vs. biological age
Age, comparisons	Continuum over years vs. arbitrary definition of elderly
Age, changes	Longitudinal design (age changes) vs. cross-sectional design (age differences)
Health status	Chronic or acute illness vs. good health; institutionalization vs. living at home
Drug therapy	Acute or chronic drug therapy vs. no drug use
Nutritional status	Good vs. poor nutrition
Environment	Smoking vs. not smoking, prior environmental exposure of elderly when young vs. current exposure in young

Source: Adapted from Mayersohn, M., Special considerations in the elderly, in *Applied Pharmacokinetics*, W. E. Evans, J. J. Schentag, and W. J. Jusko, eds., 3rd edn., Applied Therapeutics, Vancouver, WA, 1992, pp. 9-1–9-43.
Note: Some complications that need to be considered in conducting research in gerontology.

Microbiological Definition

Animals, like humans, suffer from infectious diseases ranging from those causing only mild symptoms to acute disease outbreaks with high mortality. Although some of these diseases may be partially controlled by medication, both the disease and the medication may seriously interfere with experimental work. Fortunately, most of the infectious disease of laboratory animals may be controlled simultaneously by the use of SPF techniques (Bleby, 1976).

Stocks of SPF animals were initially developed by hysterectomy of the pregnant females just prior to parturition using aseptic surgical techniques. The young were resuscitated in a building designed to prevent the entry of disease-causing organisms, or in closed isolators, and then hand-reared using a sterile milk substitute. This procedure immediately eliminates a wide range of pathogenic organisms that are normally transmitted from mother to offspring because young within the uterus are usually microbially sterile. Such SPF animals are normally free of all parasites, many viruses, and most pathogenic bacteria. Once established, such colonies breed well and can be used to supply high-quality breeding stock that does not carry the risk of introducing disease when introduced into an existing colony. Commercial breeders supply SPF mice, rats, guinea pigs, rabbits, and cats. Although some other species have been derived into SPF conditions, they are not freely available at present. Such SPF animals have (or offer) the following advantages.

A colony of animals carrying infectious pathogenic organisms is likely to be more variable than a group of SPF animals, and therefore more animals will be needed to achieve the same degree of statistical precision. For example, it has been shown that underweight mice carried five times as many parasitic nematodes as their normal-weight littermates (Eaton, 1972). Although in this case cause and effect cannot be separated, it seems reasonable to assume that such an uneven parasite burden will lead to increased variability among the experimental animals. This means that for the same statistical precision, more "parasitized" animals may be needed than if the animal were free of such parasites.

In conventional animals, there is the danger that the effects of disease may be mistaken for the actions of the experimental treatment. Vitamin A deficiency, for example, has been recorded as causing pneumonia and lung abscesses in rats, when in fact all that the deficiency is doing is to increase the severity of the infectious chronic respiratory disease found in all non-SPF rats (Lindsey et al., 1971). Pneumonia does not occur in SPF rats that are vitamin A deficient. The activation of latent disease through the experimental treatment can be extremely misleading as the control animals not subjected to the same degree of stress may be unaffected (Baker et al., 1971).

In some cases, mild infections tray mask the results of an experimental treatment. For example, rats are widely used in inhalation toxicology, yet the lesions of chronic respiratory disease may completely obscure the effects of the experimental treatment. In one case, chlorine gas caused lung lesions, but as the animals grew older, the differences between the treated and control groups were completely obscured by the chronic respiratory disease, so that the two groups became histologically indistinguishable (Elmes and Bell, 1963).

Many long-term studies require a substantial number of animals to reach old age. As fewer SPF animals die as a result of infectious disease, fewer animals need to be started in each experimental group (Lindsey et al., 1971). Thus, for long-term studies, SPF animals may be substantially more economical than conventional ones. A sidelight of this is that the use of SPF animals has advanced survivability to the point that consideration has been given to lengthening some study types (such as carcinogenicity).

Genetic Definition (Genomics)

Laboratory animals should also be genetically defined. Behavior; response to drugs; size, weight, and shape of many organs; numbers and types of spontaneous tumors; and response to antigens depend not only on the species but also on the strain of animal (Festing, 1979a). Inbred strains of mice, rats, hamsters, and guinea pigs produced as a result of at least 20 generations of brother–sister mating are readily available. They are much better experimental subjects than the more widely used outbred "white" mice and rats for most studies. As early as 1942, Strong wrote that

> It is the conviction of many geneticists that the use of the inbred mouse in cancer research has made possible many contributions of a fundamental nature that would not have been otherwise. Perhaps it would not be out of place to make the suggestion that within the near future all research on mice should be carried out on inbred animals or on hybrid mice of known (genetically controlled) origin where the degree of biological variability has been carefully controlled.

Gruneberg (1952) even went so far as to state that "the introduction of inbred strains into biology is probably comparable with that of the analytical balance into chemistry."

Here are the main characteristics of genetically defined inbred strains:

1. All individuals of a strain are genetically identical (isogenic). The genetic uniformity of inbred strains means that each strain can be genetically typed for characters such as their blood group in the knowledge that all animals within that strain will be the same. Such data are essential in many immunological and cancer research studies, and cannot be gathered in outbred stocks, where each individual is genetically unique. Isogenicity also leads to phenotypic uniformity for all highly inherited characters, and this means that the statistical precision of an experiment using these animals is increased.
2. Inbred strains are genetically stable. Once a strain has been developed, it stays genetically constant for many years. Noninbred strains may change as a result of selective forces, but such forces cannot act on inbred strains, which can only change as a result of the accumulation of mutations—a slow process. This stability means that background data on strain characteristics remain constant for long periods—allowing for the use of such information in planning experiments.
3. Inbred strains are internationally distributed. This means that experiments conducted on some of the commoner inbred strains, which are maintained in laboratories throughout the world, may easily

be confirmed at laboratories in entirely different parts of the world. Moreover, if many laboratories are working with the same strain, background data on the strain are accumulated much faster.

4. Each strain has a unique set of characteristics that may be of value in research. In some cases, a strain may have a disease that in some way models a similar condition in humans. The best known of such models are the strains with a high incidence of a particular type of cancer. The inbred mouse strain C3H develops a very high incidence of breast tumor, strains AKR and C58 develop leukemia, SJL develops reticulum-cell sarcoma (Hodgkin's disease), and some sublines of strain 129 develop teratomas. Other strains develop autoimmune anemia (NZB), amyloidosis (YBR and SJL), congenital cleft palate (A and CL), hypertension and heart defects (BALB/c and DBA/2 mice and SHR and GH rats), obesity and diabetes (NZO, PBB, and KK mice), and even a preference for alcohol when given a free choice of 10% alcohol or plain water (strain C57BL mice).

Each of these strains may be studied in order to obtain a better understanding of the disease in the mouse or rat. Once it is understood in the animal, it will be easier to understand in the human, even though it is unlikely that the conditions are exactly comparable in animals and humans. In fact, it is clear from a study of a disease such as hypertension in the rat that the cause of the hypertension in SHR and GH is entirely different (Simpson et al., 1973), emphasizing that diseases of this sort in humans can have several different causes. Obviously, in such cases, some animal models may mimic a human disease relatively closely, whereas in other cases, there is little resemblance. Table 17.17 lists some examples of the models of disease that can be found among inbred strains of mice.

Table 17.17 Examples of Disease Models and Characteristics of Interest in Inbred Strains of Mice

Character	Strain(s)
Alcohol (10%) preference	C57BL, C57BL, C57BR/cd
Aggression/fighting	SJL, NZW
Audiogenic seizures	DBA/2
Autoimmune anemia	NZB
Amyloidosis	YBR, SJL
Cleft palate	CL, A
Chediak–Higashi syndrome	SB
Hypertension and/or heart defects	BALB/c, DBA/1, DBA/2
Hyperprolinemia and prolinuria	PRO
Obesity and/or diabetes	NZO, PBB, KK, AY
Osteoarthropathy of knee joints	STR/1
Polydipsia	SWR, SWV
Resistance to myxovirus infection	A2G
Tumors	
Leukemia	AKR, C58, PL, RF
Reticulum-cell sarcoma (Hodgkin's disease)	SJL
Lung tumors	A
Hepatomas	C3Hf
Mammary tumors	C2H, C2HA-A^{vy}, GRS/A, RIII
Ovarian teratomas	LT
Induced plasmacytomas	BALB/c, NZB
Testicular teratomas	B129/terSV
Complete absence of spontaneous tumors	X/Gf
Whisker eating	A2G

Source: Festing, M. F. W., Genetic variation and adaptation in laboratory animals, in *Das Tier in Experiment*, W. H. Weihe, ed., Hans Huber, Bern, Switzerland, 1978, pp. 16–32.

Inbred strains do not meet to model any human disease in order to be of value in search. Strains can usually be found to differ for almost every characteristic studied, including many aspects of behavior, response to a wide range of drugs and chemicals, response to antigens, response to infectious agents, incidence of spontaneous diseases, and even anatomical features. These differences can be of great value in research in a number of different ways. At the most trivial level, if a scientist is studying a response to some treatment effect, it is often possible for him to find, by surveying a number of inbred strains, a strain that is highly sensitive to his experimental treatment. In some cases, this will mean that fewer animals are needed to achieve the same degree of statistical precision in future experiments. In other cases, the more sensitive strain may well show the effect sooner than resistant strains, and this may reduce the time and facilities needed to complete the experiment.

At a slightly more sophisticated level, a comparison of sensitive and resistant strains may give extremely valuable information about the mechanism of some treatment effect. For example, if two strains differ in sensitivity to a drug, it would be of great interest to know whether this is because of differences in absorption, metabolism, excretion, or target organ sensitivity. Such a study could give information that would be extremely useful in evaluating the likely effect of the drug in humans. Preferably, such studies should be carried out on two or more sensitive strains and two or more resistant strains in order to show whether the results are uniquely strain dependent or can be generalized.

Any two inbred strains will normally differ from each other at several thousand different genetic loci. However, sets of inbred strains that differ from one another at only one or a few loci have been developed in order to study in greater detail those loci that are of particular importance in biomedical research. These are known as sets of congenic strains, and most of them have been developed in order to study the major histocompatibility complex (MHC). This complex locus is responsible for a range of immunological reactions, including immune responses and graft rejection. Obviously, if two strains can be developed, which are genetically identical apart from the MHC, it becomes possible to study the MHC in detail simply by comparing the two strains. Such strains can be developed either as a result of a fortuitous mutation within an inbred strain or by deliberate breeding using convential genetic back-crossing techniques. Several hundred strains of this type have been developed, and they are not widely used in immunology and cancer research. They have undoubtedly given much insight into the biology of the mouse MHC, which in many respects is very similar to the MHC in humans. There are more than 500 known mutants and variants in the mouse, and further 100 in the rat, though in the rat many of these have now been lost. Some of these mutants appear to mimic similar mutants in several species, including humans, and may therefore be regarded as "models" of human disease. Other mutants lack an organ such as the thymus, spleen, tail, or eyes, or they suffer from some hormone deficiency or a developmental defect. Such mutants can be extremely valuable for certain types of research even though they may not resemble any human condition. A list of some of these mutants, classified by models of disease, genetic alterations and deficiencies, and biochemical and immunological polymorphisms is given in Table 17.18. There are, for example, a number of types of genetically determined obesity and diabetes that have been extremely useful as models of similar conditions in humans (Festing, 1979b). Such animals help to show up the immense complexity of the regulation of body fat via the hormonal control of a range of metabolic interactions, each of which may be controlled by regulatory mechanisms that interact with one another. Thus, the finding of a particular biochemical abnormality is no guarantee that it is the cause of the observed obesity. It is much more likely that it is a secondary effect of the primary genetic defect. However, although many of these models of obesity may have no exact counterpart in humans, they may still be useful in screening drugs with a potential for reducing obesity (Cawthorne, 1979).

One of the most important mutants causing genetic alterations or deficiencies is the athymic nude mutation in the mouse. A similar mutation has now been described in the rat (Festing et al., 1978). The thymus is essential for the full development of the immune system, and homozygous nude mice or rats are deficient in the cell-mediated type of immune response. They are of value in fundamental studies of immune mechanisms as well as in applied cancer research. This is because, lacking

Table 17.18 Examples of Mouse Mutants of Medical Interest and of Biochemical and Immunological Polymorphisms

Models of Disease	Mouse Mutants of Biomedical Interest	
	Gene	**Name**
Anemia	sla	Sex-linked anemia
	Sl	Steel
	W	Dominant spotting
Chediak–Higashi syndrome	bg	Beige
Diabetes and/or obesity	A^y	Yellow
	A^{vy}	Viable yellow
	db	Diabetes
	db^{ab}	Adipose
	ob	Obese
Inborn errors of metabolism	his	Histidinemia
	pro	Prolinemia
Kidney disease	kd	Kidney disease
Muscular dystrophy	dy	Dystrophia muscularis
	dy^{2j}	Dystrophia muscularis-2J
Neuromuscular mutants	jp	Jimpy
	med	Motor and plate disease
	qk	Quaking
	Swl	Sprawling
	Tr	Trembling
Genetic alterations or deficiencies		
Embryonic defects	t-allets	Tailless alleles
Hair absent	hr	Hairless
	hr^{rh}	Rhino
	N	Naked
Hair and thymus absent	nu	Nude
Growth hormone absent	dw	Dwarf
Resistance to androgen	Tfm	Testicular feminization
Sex reversal	Sxr	Sex reversal
Spleen absent	Dh	Dominant hemimelia

Biomedical and Immunological Polymorphisms

Polymorphism	Gene Locus
Aromatic hydrocarbon	Ahh
Pancreatic	Amy-2
β-D-galactosidase activity	Bgs
Liver catalase	Ce-1
Erythrocyte antigens	Ea-1 to Ea-7
Esterases (serum and kidney)	Es-1 to Ds-7
Friend virus susceptibility	Fv-1, Fv-2
G-6-PD regulators	Gdr-1, Gdr-2
Hemoglobin alpha chain	Hba
Hemolytic complement	He
Histocompatibility	H-1 to H-38
Immunoglobulin	Ig-1 to Ig-4

(Continued)

Table 17.18 (*Continued*)   Examples of Mouse Mutants of Medical Interest and of Biochemical and Immunological Polymorphisms

Biomedical and Immunological Polymorphisms	
Polymorphism	**Gene Locus**
Macrophage antigen 1	Mph-1
Major urinary protein	MUP-1
Phosphoglucomutase	Pgm-1, Pgm-2
Sex-limited protein	Slp
Thymus cell antigen 1	Thy-1
Thymus leukemia antigen	Tla

Source: Festing, M. F. W., Genetic variation and adaptation in laboratory animals, in *Das Tier in Experiment*, W. H. Weihe, ed., Hans Huber, Bern, Switzerland, 1978, pp. 16–32.

the cell-mediated immune response, they are unable to reject transplanted foreign tissue, including transplants of human tumors. Such transplanted human tumors usually grow, but they retain all the characteristics of human tissue. Therefore, it is possible not only to study human tumors when growing in an animal, but it is also possible to study the effect of drugs on such tumors. This is obviously of more value than having to rely simply on the study of animal tumors in animals.

The interest in mutant "knockout" and transgenic animals has blossomed since the second edition of this book. This follows the successful development of the nude and subsequent "knockout" mice as research models (Altman et al., 2013).

ENVIRONMENTAL AND NUTRITIONAL CONDITION

The need to house the defined laboratory animal in defined and stabilized environmental conditions, with a nutritionally adequate and controlled diet, is now becoming recognized. Both diet and environment can drastically alter the physiology of the animal and its response to drugs and other experimental treatments (Eben and Pilz 1967; Angel, 1969; Boyd et al., 1970; Natelson et al., 1979; Corcharan and Wong, 1987; Corcoran and Salazar, 1988). Moreover, animals obtained from a commercial breeder may well take 2 or more weeks to acclimatize to their new environment. During this period, their physiological responses may be unpredictable, depending on the difference between the two environments (Grant et al., 1971). Housing, including bedding (Heston, 1975) is often overlooked environmental factor.

SUSCEPTIBILITY FACTORS

With all the effort (and reasons behind it) that goes into obtaining a "defined" test animal with a relatively narrow range of variation in responses, what then are the components or factors that lead some animals to be more sensitive to the toxicity of agents than others?

Consideration of the problem shows that susceptibility factors fall into two large groups—intrinsic and external. The intrinsic factors include sex, stress, age, disease, physiological state (all of which were discussed in detail in the previous chapter), species variations, and strain and animal variations ("biological variation"). External or environmental factors, meanwhile, include temperature, humidity, light, and time of day (Vaccarezza and Peitz, 1960; Zbinden, 1963; Vaccarezza and Willson, 1964a,b; Safarov and Aleskerov, 1972; Vogel, 1987; Goldenthal, 1971; see the entire March issue of *Toxicologic Pathology*). Only most recently has our focus returned to the study of stress as a susceptibility factor (see Naik et al., 1970; Ehrich and Gross, 1983; Leonard, 1988).

SUMMARY

If the human population we are concerned about is such that one or more of these susceptibility factors are present in a substantial portion of the members, steps should be taken to design studies so that such individuals are adequately represented by an appropriate model in the test animal population. Barring that, or in the face of having existing data on studies performed in a standard manner, consideration should be given to these factors when attempting to predict outcome of exposures in humans.

Species Peculiarities

There are a number of quirks associated with various studies of the common species of laboratory animals used in toxicology. Many of these are not well presented in the toxicology literature, though Oser (1981) did his best to overview problems specific to the rat and Gralla (1986) has published a review of eight species-specific responses to toxicants (a modified form of which is presented in Table 17.19). Most of these peculiarities hold at least the potential to impact on study design and interpretation. Presented here are those that the authors believe should be considered in model selection for acute studies.

Species Variation

Though anyone who has had to work in biological research with intact animals should be aware of the existence of wide variability between species, examples that are specific to toxicology should be pointed out along with a comparison of species sensitivities for a number of specific agents.

The rodenticide zinc phosphide is dependent on the release of phosphine by hydrochloric acid in the stomach for its activation and efficacy (Johnson and Voss, 1952). As a result, dogs and cats are considerably less sensitive than rats and rabbits, since the former species secrete gastric hydrochloric acid intermittently, whereas the latter secrete it almost continuously. That this case is not a rare one can be quickly established by examining some data sets where we have comparative oral lethality data on several species (including humans), such as those presented in Table 17.16.

There are numerous additional examples of such species- and genetic-based differences in pharmacology (Tedeschi and Tedeschi, 1968; Altman et al., 2013). But just as important as these variations in general patterns or effect are the species-specific responses that are associated with the commonly employed animal models (Table 17.19).

Table 17.19   Species-Specific Toxic Effects

Type of Toxicity	Structure	Sensitive Species	Mechanism of Toxicity
Ocular	Retina	Dog	Zinc chelation
Ocular	Retina	Any with pigmented retinas	Melanin binding
Stimulated basal metabolism	Thyroid	Dog	Competition or binding
Porphyria	Liver	Human rat, guinea pig, mouse, and rabbit	Estrogen-enhanced sensitivity
Tubular necrosis	Kidney	Rats (male)	Androgen-enhanced sensitivity[a]
Urolithiasis	Kidney and bladder	Rats and mice	Uricase inhibition
Teratogenesis; fetal mortality	Fetus	Rats and mice	Uricase inhibition
Cardiovascular	Heart	Rabbits	Sensitivity to microvascular constriction

Source: Modified from Gralla, E. J., Species-specific toxicoses with some underlying mechanisms, In *Safety Evaluation of Drugs and Chemicals*, W. E. Lloyd, ed., Hemisphere, New York, pp. 55–81, 1986.
[a] More sensitive than humans for many agents (such as caprolactam and halogenated solvents).

Rats

Rats are commonly accepted as the best animal models in toxicology, the closest to our ideal (Gray and Addis, 1948; Oser, 1981). Table 17.20 presents a list of some of the commonly known advantages and disadvantages of rats as models for humans.

Calabese (1983) has published a good comparative review of rats as models for humans across a wide range of toxicological and biological parameters, and they should be consulted for details on these.

Mice

Mice share many advantages and disadvantages of rats as models for humans, such as an inability to vomit (i.e., no emetic response). Additionally, their small size and high metabolic rate cause the extent of many toxic effects to be exaggerated as homeostatic mechanisms are "overshot" (Gillete, 1979).

Table 17.20   Advantages and Disadvantages of the Rat as an Experimental Model for Humans

Advantages	Disadvantages
Commonly used	Anatomical
Small size	Lack of gallbladder
Minimal housing space	Yolk-sac placenta
Prolific	Multiple mammae over body surface
Short gestation	No emetic reflex
Short lactation	Fur covered
Omnivorous	Thinner stratum corneum
Dry diet acceptable	No bronchial glands
Dosing by multiple routes	Physiological
Inexpensive	Estrus and menstrual cycles
Low maintenance cost	Multiparous
Docile	Hematology
Intelligence	Obligatory nose breather
	Concentrated urine
	Limited hypersensitivity response
	Metabolic
	Purines to allantoin
	Clinical chemistry
	Enzymatic biotransformation
	High β-glucuronidase activity
	Nutritional
	Mineral requirements
	Vitamin requirements
	Ascorbic acid biosynthesis
	Histidine biosynthesis
	Behavior
	Nocturnal
	Coprophagy
	Cannibalism
	Maintenance requirements
	Temperature and humidity control
	Noise control

Guinea Pigs

The systemic immune response in guinea pigs is exaggerated. As a result, though for many immune parameters they are the best common models for humans, these animals are subject to exaggerated respiratory and cardiovascular expressions of immunocally evoked events.

Rabbits

There are no SPF rabbits currently commercially available. Rather, the animals tend not to be as homogeneous nor of as high quality as the other common laboratory animal species. Indeed, they tend to harbor a wider range of subclinical infections that show a seasonal variation in their degree of expression (animals with a visible disease problem are more common in the spring and fall), and the stress of experimentation can cause these subclinical infections to be expressed.

Also, the alterations in dermal vascular flow that accompany the changes in phases of hair growth cause marked alterations in percutaneous absorption and in so doing may alter many dermally related responses to chemicals.

Dogs

Dogs are currently the first-choice nonrodent models for toxicity studies. They are generally very cooperative. The major physiological peculiarity they have that affects toxicity testing is the ease with which they are provoked to vomit. This makes oral dosing at best impractical and at worst impossible in the case of many compounds, even if the material is encapsulated or given in diet.

Considerations of Strain

Thus far, we have focused on the differences between the different species of common laboratory animals and on how this should influence our choice of a model. But for each of the two species that are used the most in toxicology (the rat and mouse), there is the additional level of complexity caused by differences between strains.

There are three different genetic categories of strains of rodents used in toxicological research: random bred, inbred, and F hybrids (or outbred). Random bred animals are produced in large colonies where mating occurs randomly among males and females from unrelated litters. Commercially performed random breeding should not be unplanned, but rather occur in such a manner as to minimize inbreeding. Inbred animals are the result of sister–brother or parent–offspring matings. Twenty or more generations of sister–brother and/or parent–offspring mating are necessary to establish an inbred strain. Outbred animals are the results of matings between two inbred strains and are usually more vigorous than either of the parental strains. Animals within an inbred or outbred strain are essentially identical genetically, serving to remove a significant source of biological variability.

Strains also exist in the other laboratory animal species, but are generally neither as rigorously defined nor as great a concern. Thus, they have been less studied as a source of variation within toxicity studies.

That strain differences within species are a source of significant and broad differences in results has now been well established, in many cases with varying degrees of knowledge of the underlying mechanistic basis. The resistance of some strains of rabbits to atropine is believed to be due to the hydrolysis of the drug by atropine esterase, controlled by the gene A8, belonging to the group containing the gene that governs black pigmentation. As a result, resistance to atropine and black pigmentation is often associated (Sawin and Glick, 1943). Likewise, some strains of rabbits possess a pseudococaine esterase that makes their insensitivity to this drug extreme.

There are also varieties within strains. These result from various factors that in total are labeled genetic drift and can lead to significant differences in response to toxicants. An example is the resistance that some wild rats have developed to the anticoagulant rodenticides (Gratz, 1973; Zimmermann and Matschiner, 1974), requiring that new forms of rodenticides be developed.

Strain variations in response to biologically active agents arise from the same general mechanistic differences as do species differences. Although these (Hilado and Furst, 1978) pharmacogenetics have been the subject of numerous reviews (Meier, 1963a,b; Kalow, 1965; Vessel, 1969; Hathway, 1970; Moore, 1972; Lang and Vessel, 1976; Kalow et al., 2005), it is still the case that few investigators have studied the mechanisms of variation in higher animals (Becker, 1962). Since the last edition, toxicogenomics as an essential component of risk assessment has moved into the main stream (Makris and Euling, 2009; Fuchs et al., 2013).

Biological Variation

There are also individual animal-to-animal variations in temperature, health, and sensitivity to toxicities that are recognized and expected by experienced animal researchers but are only broadly understood. The resulting differences in response are generally accredited to "individual biological variation." This same phenomenon has been widely studied and observed among humans and is expected by an experienced clinician. Examples of such individual variations in human include isoniazid, succinylcholine, and glucose-6-phosphate levels and/or activities. In the first of these, "slow inactivators" are deficient in acetyltransferase, and therefore acetylate agents such as isoniazid only slowly, and are thus more liable to suffer from the peripheral neuropathy caused by an accumulation of isoniazid. At the same time, people with more effective acetyltransferase require larger doses of isoniazid to benefit from its therapeutic effects, but in so doing are more likely to suffer liver damage.

Likewise, individuals with low levels of serum cholinesterase may exhibit prolonged muscle relaxation and apnea following an injection of a standard dose of the muscle relaxant succinylcholine, and glucose-6-phosphate dehydrogenase deficiency is responsible for the increased probability of some individuals given primaquine or antipyrine to suffer from a hemolytic anemia.

ENVIRONMENTAL FACTORS

Temperature

Changes in temperature may alter the toxicity of a compound. As examples, at ambient temperatures, colchicine and digitalis are more lethal to the rat than to the frog. But the sensitivity of the frog can be increased by raising the environmental temperature of the two species. The duration of response also decreases as the temperature is raised, suggesting that a temperature-dependent biotransformation of these compounds is involved.

Temperature may include both the background environmental temperature and the internal, physiologically regulated temperature of the animal itself. Many chemicals can profoundly alter body temperature to the acceleration of reaction rates, but rather were due to alterations in the rates of physical factors, such as the absorption rate.

Keplinger et al. (1959) investigated the toxicity of 58 chemicals under different ambient conditions, including temperature. He found that many of the patterns of acute toxicity response were biphasic relative to ambient temperature, with some temperature in the ambient range being associated with a peak sensitivity in many cases.

Humidity

Selisko et al. (1963) investigated the effects of a number of environmental factors on the acute IP toxicity of nicotine to mice and found only humidity to have a significant influence. Humidity does not have a marked influence on absorption through the skin except at the extreme limits of its range (Neely et al., 1967), and the relationship between humidity and transdermal water loss in sweating animal species is not linear (Grice et al., 1972). The relationships in nonsweating species (which include all of our common laboratory species) are even more complex (Neely et al., 1967).

The physiological status of the test animal in terms of hydration can markedly influence its response to toxicants. Muller and Vemikos-Danellis (1968) showed that the LD_{50}s of caffeine and dextroamphetamine in mice were markedly affected by both ambient temperature and the animals' hydration, with caffeine showing a large potentiation of toxicity at 30°C, whereas dextroamphetamine showed much less change. At lower temperatures (22°C and 15°C), the acute toxicity of both compounds was much less influenced by hydration (Cremer and Bligh, 1969).

Environmental temperature and humidity are generally closely related and as such have frequently been considered together (Lang and Vessel, 1976). Understanding the basis for the temperature dependence of many of the actions of biologically active compounds has been an area of significant progress over the last 20 years. Belehradek (1957) successfully combined his own and other investigators' research to produce a unified theory of cellular rate processes based on an analysis of the actions of temperature. He concluded that the rate of biological processes is primarily dependent on the resistance of cellular matter to the free movement of molecules within the cells rather than the rate of actual chemical reactions themselves. He was enthusiastic about the relationship between the rate responses of cellular systems and Slotte's temperature–viscosity relationship formula. Brody (1964) has, however, reviewed the applicability of this last to vertebrate animals and pointed out its shortcomings.

The relationship between responses to toxicants and ambient temperature in animals is sometimes paradoxical. Usinger (1957) investigated this in mice and found a series of biphasic relationships with "optimal," or peak, ranges. Mean oxygen consumption per unit body weight diminishes as temperature increases further. Likewise, he measured the rectal temperatures occurring when the ambient temperature was 25°C, increasing on either side of this temperature.

Ahdaya et al. (1976) investigated thermoregulation in mice exposed to parathion, carbaryl, and DDT at temperatures of 1°C, 27°C, and 38°C. All three of the pesticides were found to be least toxic at 27°C. Doull (1972) has reviewed these temperature-dependent responses for many chemicals and presented the hypothesis that temperature is directly correlated with the magnitude and inversely correlated with the duration of the biological response to biologically active xenobiotics in many organisms. Though this temperature dependence stands as a general rule, there are a number of special case exceptions. And it is clear that the effect of temperature on one response variable may not necessarily be predictive of the effects on other biological response variables.

Baetjer and Smith (1956) found that the onset of death, rate of dying, and rate of recovery due to parathion in mice were more rapid, whereas the mortality was higher at 35.6°C than at 22.8°C. At 15.5°C, the onset of death was delayed and the total mortality was greater than at 22.8°C. They also investigated the influence of both pre- and postexposure temperatures on the response of the mice and determined that mortality varied directly with the preexposure ambient temperatures and inversely with the postexposure. Their conclusion was that the results could not be attributed.

Barometric Pressure

Interest in the effect of atmospheric pressure on the toxicity of chemicals is fairly recent, arising from human activities in space and deep-sea diving vessels. At high altitudes, the toxicity of digitalis and strychnine is decreased, whereas that of amphetamine is increased. The influence

of atmospheric pressure seems to be mainly (but not entirely) attributable to altered physiological oxygen tension rather than a direct pressure effect (Brown, 1980). Recently, this interest has taken a new turn as concern as to the possible hazard of fires and atmospheric contaminants on submarines has surfaced.

Light

Whole-body irradiation with electromagnetic radiation, including light, increases the toxicity of central nervous system (CNS) stimulants and decreases that of CNS depressants. The toxicity of analgesics such as morphine does not seem to be altered. Many toxicants exhibit a diurnal pattern of response in animals that is generally related to the light pattern. In rats and mice, P-450 enzyme activity is at its greatest at the beginning of the dark phase of the cycle.

Social Factors

A variety of social factors (interactions between individual animals and between animals and research workers) can modify the toxicities of chemicals in animals and undoubtedly also in humans. (Table 17.21) Animal handling, housing (singly or in groups), types of cages, and laboratory routine are all important components of such considerations.

Edward (1982) should be consulted for a good overview of the factors to be considered in the design and operation of a laboratory in terms of both good science and economic and regulatory considerations.

Temporal Factors

Most biological organisms are influenced, directly and/or indirectly, by a stream of daily and annual variations in their environment. These variations include light, temperature, social interactions, and food and have resulted in notable cyclical variations in function. Table 17.9 presents some of the better established of these.

Many of these cycles can be considerably amplified and/or modified. Liver function, for example, includes liver glycogen (which is also seasonally variable), glycogen phosphorylase, tyrosine transaminase, tryptophan pyrrolase, and esterase; some of these rhythmic changes are reflected in hepatic cell ultrastructure. Renal function includes urine volume and pH and excretion of sodium, potassium, chloride, phosphate, uric acid, adrenal cortex, and probably other tissues as well. Blood

Table 17.21   Psychological Functions Showing Time-Based Cyclical Variations

Activity/sleep
Body temperature
Pain threshold
Adrenocortical function
Skin histamine sensitivity
Liver function
Renal function
Eosinophil count
Mitotic rates
Food consumption
Body weight

Sources: Halberg, F. et al., *Proc. Soc. Exp. Biol. Med*. 103, 142, 1960; Lenox, R. H. and Frazier, T. W., *Nature* 239, 397, 1972; Reinberg, A. et al., *Int. J. Chronobiol*. 1, 157, 1973; Scheving, L. E. et al., *J. Eur. Toxicol*. 7, 203, 1974.

leukocytes other than eosinophils show daily variation, although the eosinophilic changes have probably been the most studied. Reviews of these topics are found in Reinberg and Ghata, 1964; Aschoff, 1965; Bunning, 1967; Conroy and Mills, 1970; Mills, 1973; and Gall, 1977.

Many unicellular organisms also show cyclically varying functions, for example, cell generation time, photosynthetic capacity, phototaxis, and luminescence. In higher plants, growth, leaf and petal movement, and CO_2 fixation are rhythmic functions, and in fungi, spore discharge (Wilkins, 1973).

An interesting feature of these rhythms is that they continue when rhythmic external stimuli are removed. If the daily light/dark cycle is turned into continuous light and if temperature variation is suppressed and if, in humans, clues like watches and clocks and fixed meal times are removed, the rhythmic variation in function continues, but with a period that may differ slightly from the original 24 hours. This is the so-called free-running period. A 24-hour rhythm may become, for example, 25.0 or 22.9 hours (usually, however, between 20 and 28 hours) and settles to this new period indefinitely until external clues are restored. This phenomenon is the origin of the expression "circadian," meaning about a day's length.

Considerable work has gone into trying to identify the biological clock that maintains these rhythms, so far without clear-cut success. Does the clock reside in an organ (for example, the CNS or the adrenal cortex), or is it a cellular function? The answer is probably both: that the cell has mechanisms that allow it to entrain to environmental rhythms and that in higher organisms, these become systematized in regulatory organs to coordinate the functioning of the body as a whole in response to environmental changes (Mills, 1966, 1973).

Both unicellular organisms and avian and mammalian cells in culture show circadian rhythms in the absence of exogenous influences (Bruce, 1965). Present arguments center around whether the cellular clock is based on sequential DNA transcription or to biochemical networks with natural oscillatory periods. A more recent hypothesis suggests that the cell membrane, with its stable lipids and mobile protein ionic gates, may serve as an oscillator with the underlying slow periodically (Njus et al., 1974).

The majority of the laboratory animals used in toxicology are nocturnal, and they do not change while in the laboratory. Our procedures rarely take the fact and implication of this underlying biological activity rhythm into account.

Diurnal rhythms (more correctly nychthemeral; diurnal refers to daytime, nocturnal to nighttime) are probably the most important, but there are almost certainly other cyclical changes in physiological function, connected to season or to sexual function, which for the most part we disregard and which may occasionally be important.

The significance of circadian variation for drug action and toxicology will depend very much on the nature of the drug, its absorption characteristics, and the way in which it is administered. Toxicity from continuous atmospheric exposure is not likely to show much circadian dependence, nor from a drug only slowly absorbed or slowly metabolized to an active component. But these are perhaps the exceptions, and with the increasing range of drugs showing some clinical effectiveness, many of which also carry undesirable side effects, it would seem sensible to administer these in a regimen that allows them to exert their maximum desired effect with a minimum of side effects. While drugs acting on the CNS, the cardiovascular system, and the kidney and steroids are obvious candidates for such consideration, there is evidence that susceptibility to infection is also circadian dependent and chemotherapeutic agents and the antibiotics may not be outside such an inquiry.

In addition, a sizeable proportion of the population has its biological clock thrown into potential disarray. Besides the pilots and aircrew of long-distance flights and the jet-set traveler, the night-shift work force is numerous enough and includes not only the factory workers and truck drivers but hospital staff and watchkeepers at sea, all carrying considerable responsibility. If there is a relationship between drug action and the circadian cycle, it deserves proper evaluation.

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Laws, Regulations, and Guidelines for Animal Care and Use in Research

Charles B. Spainhour

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INTRODUCTION

An individual was once quoted as stating "It is harder to perform research in animals than it is in humans." This is probably the same thought that many of us have also had at least at one time or another. What he was referring to were the multiple and varied requirements, rules, and regulations that had to be put in place and closely followed when setting up an animal care and use program. And these efforts all had to be completed prior to actually procuring and then using animals in research projects. Many countries have a specific doctrine on the use of animals in research, some of which involve a plethora of rules and regulations that require a great deal of administrative time and effort, while others have very basic requirements. The requirements for the use of animals in research range from guidelines to laws that must be followed and are enforced. If the laws are not followed, there are serious repercussions ranging from significant monetary fines to incarceration. Guidelines on the other hand are typically voluntary but many are strongly suggested. Voluntary guidelines provide facilities with the standards and information necessary on how to attain what is considered to be the "gold standard of animal care and use." This chapter will provide the reader with an overview of the laws, regulations, and guidelines for the care and use of animals in a research setting as well as resources that will provide more in-depth information to the reader.

HISTORY AND BACKGROUND

Animals have been used in research for thousands of years, and often the procedures performed on animals when they were first used to gain an understanding of how living systems functioned were essentially brutal and inhumane. The Greeks studied anatomy and physiology by using the dead as well as living animals. In order to determine how the body functioned, surgical procedures were performed on fully awake animals to see how blood flowed, how the heart beats, etc. Of course, early humans did not have access to anesthetics and many did not feel that animals perceived pain as we do because they were assumed to be inferior creatures. Experimentation on live animals was justified as a "path to the truth" (Brewer, 1999). To put things in perspective, one must also remember that surgical procedures at this time were also being performed on humans without anesthesia and that learning anatomy and physiology from animals helped early healers to better understand how to help humans and understand physiologic processes and diseases. The development of anesthesia occurred in the middle of the nineteenth century as well as an evolution out of the dark ages that led to an era of inquiring minds wanting to know and with it came an increase in the use of animals in biomedical research. Initially, animals were being used by researchers that had no idea of how to properly care for animals or the complications that various disease entities could have on their research. Proper animal care began to evolve from researchers hiring "animal caretakers" who genuinely cared about the animals and their health. These individuals were the predecessors to our modern animal care technicians.

With the realization of the progress and knowledge that could be gained from the study of animals in relationship to human conditions and diseases, the use of animals in research became popular, which in turn led to an increase in the number of animal colonies that were being established for research purposes. Researchers began to realize that in addition to choosing the correct animal models for the type of research they were performing, they also needed healthy subjects. As the use of animals in research became widespread and animal facilities at prestigious institutions such as the Royal Prussian Institute for Experimental Therapy, Harvard, Johns Hopkins, and the Mayo Clinic were being established, the development of proper husbandry for specific species being housed and the importance of disease control and elimination of disease in animal colonies were advancing (Brewer, 1999). Institutions began hiring veterinarians, who often were already involved in various research projects at the institution, to manage their animal colonies. The desire to properly

care for and humanely use research animals basically began with veterinarians, animal caretakers, and researchers. This is the group that started the first organization for the humane care and use of research animals, the Animal Care Panel (ACP). The ACP was founded in 1950 and it held its first meeting on the same year; it would later become the American Association for Laboratory Animal Science (AALAS). The ACP prepared numerous publications on the proper care and use of laboratory animal species. The first publication, *Standards for the Care of the Dog Used in Medical Research*, was published in 1952 and many more publications followed including the first *Guide for Laboratory Animal Facilities and Care*, *1963* (Mulder, 1999). The ACP also implemented training courses for animal care technicians. The Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) also evolved out of the ACP in 1965 and encouraged institutions to voluntarily become accredited in order to promote the establishment and development of high-quality animal care programs. In addition to these organizations, others such as the Institute of Laboratory Animal Resources (ILAR) (Wolfle, 1999) and the American College of Laboratory Animal Medicine (ACLAM) (Middleton, 1999) were established in the 1950s to also promote the proper care and use of laboratory animals. Other programs that involved training and the development of new and improved husbandry techniques in support of developing improved laboratory animal care and dissemination of that information were also being established in the late 1950s and early 1960s but are beyond the scope of this chapter to discuss in further detail.

It was not until 1966 that the United States developed federal regulations to "protect" research animals. Advocates against the use of animals in research often use the media to fuel their campaigns as was the case in 1966. In response to the public outcry ignited by an article published in a 1966 issue of *LIFE* magazine on the inhumane care of dogs by dealers selling them for research, legislature for the protection of animals that were to be used in research was enacted (Schwindaman, 1999). The U.S. Department of Agriculture (USDA) was charged with the oversight and enforcement of the Laboratory Animal Welfare Act (LAWA). The USDA had to develop minimum standards for the care and management of laboratory animals. USDA consulted with a multitude of various groups to establish the minimum standards of animal care. The initial LAWA only required those facilities that were using dogs and cats for research and received federal funds to register with the USDA. Furthermore, only dealers that bought and sold dogs and cats and conducted business over state lines were required to be registered. The LAWA was amended in 1970, changing the name to the Animal Welfare Act (AWA), 1979 and 1985. Each amendment broadened the scope of items, species, and groups that were covered by the AWA as well as the regulatory and enforcement responsibilities of the USDA. The 1985 amendments brought the greatest changes and accountability to research facilities in the United States. The amendments required the conduct of a literature search for alternatives prior to the performance of any research procedure that had or had the potential to cause more than the presence of momentary pain or distress to research animals, established an Institutional Animal Care and Use Committee (IACUC), required the definition of a program for the use of pain- and stress-relieving measures as necessary, identified the institutional official (IO) as having the accountability and responsibility for meeting the AWA requirements, established the importance of exercise programs for dogs, required environmental enrichment programs for nonhuman primates (NHPs), put in place a program where USDA inspectors mandatorily visit facilities unannounced at intervals of a minimum of once each year, compiled a list of criteria that an IACUC must use to evaluate protocols, established training procedures for personnel working with animals, and established the Animal Welfare Information Center (AWIC) (Schwindaman, 1999). Since 1985, there has not been much new formal legislative activity concerning the AWA other than the Pet Theft Act of 1990 that requires animal shelters to hold dogs and cats for at least 5 days prior to selling them to dealers and the recent January 30, 2013, amendment Handling of Animals: Contingency Plans (*Federal Register*, 2012). Dealers must provide to any research facility to which they sell the dog or cat the source and origin of the dog or the cat, and a contingency plan on possible disasters that may affect a facility must be formulated and written, and employee

training on the plan must be implemented. Other legislative bills that are being considered include the Pet Safety and Protection Act of 2011 (H.R. 2256, 2011) and the Great Ape Protection and Cost Savings Act of 2011 (S.810, 2011). The Pet Safety and Protection Act of 2011 proposes the elimination of dog and cat dealers that obtain dogs and cats from random sources such as private breeders, pounds, and individuals relinquishing pets. The USDA has classified dealers who market random source dogs and cats as Class B dealers. This bill would virtually eliminate the use of random source dogs and cats by research facilities. Currently, the majority of large breed dogs often used in cardiovascular and orthopedic research are obtained from Class B dealers as there are very few dealers who breed animals specifically for research, also known as Class A dealers who can provide these animals in any quantity. Also, the pool of Class A cat breeders is currently severely limited considering there is only one major breeder remaining. Unfortunately, this bill has been introduced into the legislature every year since 1996 and fortunately still has not passed. The Great Ape Protection and Cost Savings Act of 2011 has come very close to becoming law. It prohibits the use of chimpanzees in "invasive" research since these are the only great apes currently used in laboratory research settings. The definition of invasive outlined in this act precludes the use of chimpanzees in any type of biomedical research. If this bill becomes law, it will impede research on conditions and diseases for which chimpanzees are judged to be the closest model for the condition or disease in humans such as in infectious disease research involving entities such as hepatitis B, hepatitis C, and AIDS (Committee on Long-Term Care of Chimpanzees, 1997). The National Institutes of Health (NIH) is currently providing testimony to Congress on the need for chimpanzees in biomedical research albeit on a limited basis. The NIH is attempting to reach a compromise that will alter the stringent wording in the current act that will only allow the use of chimpanzees in research on a very specific as-needed basis requiring a very strong scientific justification for the use of the animals. These are the main acts that are being presented to the federal government that currently affect biomedical research using animals, but there are countless legislative bills at various levels of government (state and local) that are being considered or passed that deal with animal welfare and rights (National Association for Biomedical Research [NABR], n.d.; Pennsylvania Veterinary Medical Association, n.d.). Many of these potential bills are being presented and advocated by animal rights (AR) groups such as the Humane Society of the United States (HSUS), People for the Ethical Treatment of Animals (PETA), and Physicians Committee for Responsible Medicine (PCRM).

Laws, regulations, and enforcement agencies that govern animal welfare have been established worldwide. In Europe, animal welfare laws are set by the European Union (EU) (The European Parliament and the Council of the European Union, 2010). The EU is made up of various countries in Europe and each country can have its own animal welfare laws, regulations, and enforcement agencies, but they are expected at a minimum to comply with the regulations established by the EU. For example, Italy is advocating some legislative actions that are much more restrictive than the EU requirements. The Italian government is proposing a ban on the breeding of dogs, cats, and NHPs for research purposes, prohibiting the performance of any experiments that may cause pain without using anesthesia and stricter government oversight on the use of transgenic animals (Nosengo, 2012). The EU has banned the use of great apes in research and the testing of cosmetics in or on animals (Commission of the European Communities, 2004). In Canada, animal welfare guidelines are established by the Canadian Council on Animal Care (CCAC) (1980, 1984, 1998, 2010). Japan's animal welfare requirements can be found in the Law for the Humane Treatment and Management of Animals and Standards Relating to the Care and Management of Experimental Animals. These are a few examples of the animal welfare requirements of various countries. As the laws, regulations, and guidelines of various countries are reviewed, one can appreciate the range of animal welfare advocacy and anticipate that the further protection of animals is accelerating at a rapid pace.

U.S. ANIMAL LAWS AND REGULATIONS

Animal Welfare Act

The AWA contains the details of the federally mandated laws and regulations governing animal welfare in the United States. The USDA is the government agency that was chosen by Congress to formulate the regulations for compliance with the AWA and the enforcement of the regulations (Schwindaman, 1999). Under the AWA, research facilities are required to register with the USDA if they use any AWA-covered species in research, tests, teaching, or experiments. Research facilities are required to renew their registration status every 3 years. Species that are not covered by the AWA are birds, mice (genus *Mus*), and rats (genus *Rattus*) bred for use in research; horses not used for research purposes; farm animals, including livestock and poultry, used or intended for use as food or fiber or in agricultural research; and fish and invertebrates (crustaceans, insects) (United States Department of Agriculture, 2006). The latest version of the AWA and Animal Welfare Regulations (AWR) was published in August 2002 by the USDA, Animal and Plant Health Inspection Service (APHIS) (United States Department of Agriculture, 2002). This document contains the requirements of the AWA as well as the regulations followed by the USDA to ensure sound animal welfare. Additional documents used by the USDA for guidance on how to interpret and carry out the AWA and its regulations are the Animal Care Resource Guide Policies (United States Department of Agriculture, 2011) and the Consolidated Inspection Guide (United States Department of Agriculture, 2012). The species specifically covered in the AWR include dogs, cats, rabbits, guinea pigs, hamsters, NHPs, marine mammals, and then a general category of all other warm-blooded animals. The subsections for each species include Facilities and Operating Standards, Animal Health and Husbandry Standards, and Transportation Standards. Minimum requirements for cage size can be found in the Facilities and Operating Standards section for each species and in Table 18.1.

In addition to the space requirements for the various species covered in the AWR, temperature, lighting, ventilation, facility structural requirements, waste disposal, feeding, bedding/caging surfaces, sanitization, watering, housing (single vs. group), etc., requirements are covered for each species. Facilities are now also required to have a disaster plan in place as well as an employee training program on how disasters should be handled as stipulated in the facility disaster plan. The items covered in the regulations are mandatory and not elective requirements. Transportation requirements for each species are comprehensively covered including how many animals may be transported in the same container (e.g., no more than 15 rabbits in one primary container), container ventilation requirements, structure of the transport container, how often animals need to be fed and watered on a trip, how often animals need to be observed while in transit, and handling of the transport containers when they hold animals.

In addition to the basic care and handling of the species covered by the USDA, two laboratory species have additional requirements. Along with the mandatory minimum floor space requirement, the dog must be given the opportunity to exercise. This exercise cannot be forced such as putting the dog on a treadmill, rather the dog must be given additional floor space to exercise and play. The methods and frequency of the opportunity to exercise are delegated by the facility. The facility along with the attending veterinarian (AV) must draw up a plan to provide the dogs with the opportunity to exercise. When exercising, the available floor space must be twice the floor space of the minimum required floor space. Many facilities have various methods for providing dogs with the opportunity to exercise, and some of these methods include walking on a leash, allowing dogs to run around on the floor of an animal room for a set amount of time, pair housing in caging that at a minimum meets the floor space requirement of each dog thereby doubling the floor space when the dogs are together (e.g., having a sliding door between two primary enclosures and opening the doors to allow the dogs to comingle), and making indoor or outdoor pens. If dogs are group housed and the enclosure floor space equals the floor space required

Table 18.1   AWR Requirements for Minimum Space of Primary Enclosures (August 2002)

Species	Weight	Floor Area/Animal	Cage Height[a]	Comments
Dog	NA	(Dog length [in.] $\pm$ 6)^2 144	6″ above the head of the tallest dog in the enclosure	Bitches with nursing puppies must be provided with additional floor space. Generally, each puppy should get a minimum of 5% of the minimum floor space requirement of the bitch.
Cat	$\leq$4.0 kg	3.0 ft^2 (0.28 m^2)	24″ (60.96 cm)	Queens with nursing kittens must be provided with additional floor space. Generally, each kitten should get a minimum of 5% of the minimum floor space requirement of the queen.
	>4.0 kg	4.0 ft^2 (0.37 m^2)	24″ (60.96 cm)	
Guinea pig	<350 g	60 in.^{2} (387.12 cm^2)	7.0″ (17.78 cm)	
	$\geq$350 g	101 in.^{2} (651.65 cm^2)	7.0″ (17.78 cm)	
	Female + litter	101 in.^{2} (651.65 cm^2)	7.0″ (17.78 cm)	
Hamster	<60 g	10 in.^{2} (64.52 cm^2)	6.0″ (15.2 cm)	
	60–80 g	13 in.^{2} (83.88 cm^2)	6.0″ (15.2 cm)	
	80–100 g	16 in.^{2} (103.23 cm^2)	6.0″ (15.2 cm)	
	>100 g	19 in.^{2} (122.59 cm^2)	6.0″ (15.2 cm)	
	Female + litter	121 in.^{2} (780.45 cm^2)	6.0″ (15.2 cm)	
Rabbit individual	<2 kg	1.5 ft^2 (0.14 m^2)	14″ (35.56 cm)	
	2–4 kg	3.0 ft^2 (0.28 m^2)	14″ (35.56 cm)	
	4–4.5 kg	4.0 ft^2 (0.37 m^2)	14″ (35.56 cm)	
	>5.4 kg	5.0 ft^2 (0.46 m^2)	14″ (35.56 cm)	
Females + litters	<2 kg	4.0 ft^2 (0.37 m^2)	14″ (35.56 cm)	
	2–4 kg	5.0 ft^2 (0.46 m^2)	14″ (35.56 cm)	
	4–4.5 kg	6.0 ft^2 (0.56 m^2)	14″ (35.56 cm)	
	>5.4 kg	7.5 ft^2 (0.70 m^2)	14″ (35.56 cm)	
NHPs[b]				
Group 1	<1 kg	1.6 ft^2 (0.15 m^2)	20″ (50.8 cm)	
Group 2	1–3 kg	3.0 ft^2 (0.0.28 m^2)	30″ (76.2 cm)	
Group 3	3–10 kg	4.3 ft^2 (0.40 m^2)	30″ (76.2 cm)	
Group 4	10–15 kg	6.0 ft^2 (0.56 m^2)	32″ (81.28 cm)	
Group 5	15–25 kg	8.0 ft^2 (0.74 m^2)	36″ (91.44 cm)	
Group 6	>25 kg	25.1 ft^2 (2.33 m^2)	84″ (213.36 cm)	

[a] From cage floor to cage top.
[b] The different species of NHPs are divided into six weight groups for determining minimum space requirements, except that all brachiating species of any weight are grouped together since they require additional space to engage in species-typical behavior. Examples of types of NHPs in each group: Group 1, marmosets, tamarins, and infants (less than 6 months of age) of various species; Group 2, capuchins, squirrel monkeys and similar size species, and juveniles (6 months to 3 years of various species); Group 3, macaques and African species; Group 4, male macaques and large African species; Group 5, baboons and nonbrachiating species larger than 15 kg; and Group 6, great apes over 25 kg.

for each dog or an individually housed dog has double the required minimum floor space, additional opportunity to exercise is not required. Dogs can be exempted from exercise by order of the AV for reasonable medical or behavioral reasons. If the animal is exempt from exercise, the AV needs to reevaluate the animal every 30 days to see if exercise can be reinstated. If the AV determines that the animal has a permanent condition that is not conducive of exercise, this needs to be completely and thoroughly documented and the opportunity to exercise will not be required. Dogs may also be exempt from exercise based on an IACUC-approved scientific protocol that has "adequate" scientific justification for the exemption. Records for any exemptions must be maintained and reported on the USDA annual report.

The second common laboratory animal species that have special requirements are NHPs. An environmental enrichment/enhancement program must be in place to promote the psychological well-being of NHPs, and it is essential that this be developed by the individual research facility. Facilities that house NHPs are advised to consult publications and organizations that provide information on the needs of various species of NHPs and tested and tried methods of providing environmental enrichment/enhancement to meet these needs as the basis of their programs (Committee on Well-Being of Nonhuman Primates, 1998). Social housing is considered one of the mainstays of psychological enrichment in NHPs and is now strongly advocated by the USDA. In the past, individual housing of NHPs in research facilities was common practice; and food consumption measurements, odd number of animals in dose groups, transfer of test article between animals, injury potential to both the animals and staff when social housing animals, etc., were reasons that were considered acceptable. In 2010, these reasons were no longer considered acceptable. Dose groups with odd numbers of animals should be housed in triplicate or greater with one strategy being using tunnels on the front of the cages to allow bottom animals to also use top cages and vice versa. The Food and Drug Administration (FDA) accepts group housing data such as group observations of fecal and urine output and group food consumption and does not seem concerned with potential test article transfer as long as animals from the same dose group are housed together. Animals can be separated for dosing procedures by closing off individual cages or using innovatively designed dosing cages where the NHPs are trained to enter a dosing cage, removed from the common pen, dosed, and returned to the common area. There are some exceptions to the rules of group housing, and some of these include the presence of an overly aggressive or vicious animal or a debilitated animal. For animals that are overly aggressive, extensive documentation needs to be available to support and verify that there have been multiple attempts that failed to work before an animal can be labeled in such a manner. Other exceptions include animals presenting with signs of a contagious disease; unless the whole group has it, then they could be group housed if they are not debilitated or are noncompatible. NHPs also are required to have environmental enrichment. The enclosure environment must be enriched by providing a means for the NHP to express species-specific behaviors. Many items are being used by a variety of facilities in order to provide enrichment to NHPs. Some of these items such as foraging boards, perches, mirrors, toys, televisions, and treats are available commercially. There are even more items that innovative facilities have invented on their own, and many of these items and ideas are shared at various laboratory animal meetings and on websites or list servers. Similar to the exemption of exercise in dogs, exemptions from participation in environmental enhancement for NHPs are allowed. Veterinary exemptions can be made, must be documented, and must be reviewed every 30 days. Permanent exemptions can be made with an appropriate level of detailed documentation. Exemptions can be protocol driven with appropriate scientific justification and IACUC approval. Exemptions due to research requirements must be reported yearly on the annual report. NHPs do not have to be exempt from all aspects of the environmental enrichment program. For example, an NHP that cannot be group housed can still be allowed access to activities that permit enrichment of their environment and actually be exposed to more types of enrichment or complex enrichment in an attempt to make up for the lack of social housing.

In addition to animal care standards, the AWR requires research facilities that are subject to the AWA to establish an IACUC. IACUC requirements as stipulated by the regulations will be covered in

the section of this chapter on IACUCs. Other requirements for research facilities include the proper training of employees that care for, handle, and/or use animals in research, a program for the provision of adequate veterinary care, detailed record-keeping requirements and maintenance (records must be maintained for 3 years), the mandatory completion and submission of an annual report to the USDA, and the completion or righting of deficiencies as identified and specified during the course of an inspection being conducted by a USDA inspector. Each research facility must employ an AV. The veterinarian can be full time or part time. If the veterinarian is part time, then a written program of veterinary care must be developed between the veterinarian and the research facility. All animals must be observed at a minimum of once daily by adequately trained personnel in order to assess their health and well-being. There must be a mechanism for the direct and timely communication with the AV if animal health issues develop. Each research facility is required to fill out an annual report on the use or intended use of AWA-covered species. The time frame for the annual reporting period for the USDA is from October 1 to September 30 of the following year, and the report is due by December 1. The annual report includes statements that assure that animals are being properly used in research. The number of animals being housed and used at the research facility between the dates stated earlier must be documented in the annual report. Animals are placed in one of four categories. Category B should include animals that are being held for research, teaching, testing, etc., but on which no procedures have been conducted. Animals placed in Category C have been used for procedures that cause no more than momentary pain or distress such as blood collection and routine injections. Category D should contain animals that have had procedures performed on them that would cause more than momentary pain or distress but were administered the appropriate anesthetics, analgesics, or tranquilizing drugs (e.g., surgical models). Animals placed in Category E had procedures performed on them that caused more than momentary pain or distress but had appropriate anesthetics, analgesics, or tranquilizing drugs withheld as the administration of these substances would interfere with the procedure being performed, the results, or the interpretation of the data. Examples of such studies are experiments that use pain models, models of inflammation, chronic disease models, etc. If a research facility has animals that are in pain Category E, a detailed explanation of why the alleviation of pain or distress could not be provided needs to be attached to the annual report. Exemptions of dogs from exercise and NHPs from the environmental enrichment program that are required by IACUC-approved protocols must also be included on the annual report.

The USDA is required to inspect all registered research facilities to be in compliance with the AWA and its regulations at a minimum of once a year if not more frequently. These unannounced inspections are conducted by veterinary medical officers or other trained personnel such as veterinary technicians who are designated as animal care inspectors. The inspector will conduct a detailed walk through the facility to assure that animal care is in compliance with the regulations, the structural aspects of the facility (i.e., caging, equipment, walls, flooring, doors, walls) are in satisfactory condition, and all paperwork is thorough and complete and has been performed in a timely fashion. If they are inspecting a research facility, the IACUC minutes, membership, attendance, deliberations, etc., are closely scrutinized for compliance. Research facilities must furnish the inspector in a timely fashion with any information or records requested that the facility is required by the AWA and its regulations to maintain. The inspector has the authority to make copies of these records and take photographs of any areas of noncompliance. Regardless of any standard operating procedures (SOPs) that a facility may have in place with regard to the use of cameras, the USDA inspector is permitted because of his or her authority to take such pictures for documentation. However, the facility should completely understand why the picture is being taken, make sure that the inspector takes a photograph of the item of concern only and not just a broad photograph, and finally request a copy of all photographs taken. At the end of the inspection, the inspector will write up an inspection report. Any noncompliant items (NCIs) will be enumerated in the report. The section and subsection of the AWA and AWR will be cited along with the pertinent NCI on the inspection form. The report is signed by the inspector and the responsible member or suitable designate of the research facility. If the responsible party of the

facility does not agree with the inspector's findings, there is an appeals process that can be followed. If a facility wins the appeal, an amended USDA inspection will be filed by the USDA. All inspection reports are posted on the web and available for anyone to read and study.

The year 2010 was declared the "Age of Enforcement" by the deputy secretary of the Department of Agriculture, and along with this declaration was a substantial increase in the monetary value of fines to be assessed as well as the "two strikes and you're out" rule (Bennett, 2012). If a facility has two NCI items that are classified under the same section of the AWA, the facility may now be considered to be in violation of the AWA. This in turn can lead to the levy of significant fines. It is important to recognize that recurrent findings do not have to be consecutive nor do they have to occur in the same area of the facility or the same administrative function. It is imperative that research facilities have a well-managed animal care program, facilities, and inspection management program and have the support of the facility administration up to the highest levels of management to provide the necessary resources to avoid the discovery and citations of NCIs and potential fines.

Office of Laboratory Animal Welfare

The Office of Laboratory Animal Welfare (OLAW) is the division of the Public Health Service (PHS) that reviews an institution's proposed program of animal care and use in PHS-conducted or PHS-supported activities (Office of Laboratory Animal Welfare, 2002). If the proposed program of animal care and use meets PHS requirements, OLAW will issue the institution an assurance number. The PHS is the parent organization of the NIH, which is the branch to which researchers typically submit their grant applications in order to secure funds for their research. If an institution does not have an OLAW assurance number, no NIH grant–supported research can be conducted at the facility. In order to obtain an assurance number from OLAW, the institution must submit its proposed institutional program for animal care and use. The institution's animal care and use program must use the most recent version of the *Guide for the Care and Use of Laboratory Animals* ("guide") (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011) that has been approved by OLAW for the basis of developing and implementing an institutional program for activities involving animals (United States Department of Health and Human Services, n.d.). The program description requirements can be found in the *Public Health Service Policy on Humane Care and Use of Laboratory Animals.*

Institutions are placed by OLAW into one of two categories, which then determine the documentation that will be required by OLAW for obtaining an assurance and assurance number. Category 1 institutions are those who are currently accredited by the AAALAC International. These institutions do not have to submit their most recent semiannual IACUC evaluation reports with the assurance application. Category 2 institutions are not AAALAC accredited and can rely only on their IACUC to evaluate their animal care and use program. These institutions are required to send the most recent semiannual report into OLAW with the assurance application. OLAW can also perform special reviews and/or a site visit in order to further assess the facility's compliance with the requirements of the PHS policy.

Once an assurance number has been obtained, facilities are required to keep records of items such as copies of the approved assurance, IACUC minutes, proposed projects and subsequent IACUC deliberations on research proposals, minority opinions on research to be conducted, semiannual IACUC reports, and any records of accrediting body inspections. All records must be maintained for a period of at least 3 years. Records for ongoing approved proposals must be maintained until the activities on the proposal are completed plus 3 years beyond that date. Assured facilities must submit an annual report to OLAW in writing through the IO, and the report must include any changes in the AAALAC accreditation status (or any other acceptable accreditation), any changes in the institution's program for animal care and use, any changes in

IACUC membership, and disclosure of any and all dates that facility inspections and program reviews were performed by the IACUC. If there are no changes in any of these points, this must be stated in writing by the IO to OLAW. If there are any serious or continuing noncompliance issues with the PHS policy, serious deviations from the "guide," or suspension of an activity by the IACUC, they must be reported to OLAW. Minority opinions expressed by IACUC members must be disclosed and filed also.

Applications for grant monies from the NIH must be submitted through OLAW if animal use is involved. The applications must contain a section describing the care and use of the species that are being proposed for use. The required information includes the identification of the species and the numbers of animals for the program, a statement on the rationale for the selection and use of the animals as well as statements on the appropriateness and relevance of the species chosen and numbers of animals requested, a complete detailed description of the proposed use of the animals, a detailed description of procedures to be used to minimize pain and distress if it occurs, and methods of euthanasia. The proposal must have IACUC approval. The IACUC is expected to review and assess the protocol, taking into consideration the requirements of the U.S. Government Principles that can be found in the PHS policy manual, August 2002, and Table 18.2. IACUC approval can be filed any time prior to the grant and approval is good for 3 years. If IACUC approval is subsequent to the submission of the proposal, any modifications required by the IACUC must be verified. The verification of IACUC approval must be signed by an individual authorized by the institution. Grant awards will not be released until IACUC approval is verified.

Table 18.2   U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training

The PHS policy implements nine U.S. Government Principles that are the foundation for humane care and use of laboratory animals in this country. These principles were developed by the Interagency Research Animal Committee and adopted in 1985 by the Office of Science and Technology Policy:

  I. The transportation, care, and use of animals should be in accordance with the Animal Welfare Act (7 U.S.C. 2131 et. seq.) and other applicable federal laws, guidelines, and policies.

 II. Procedures involving animals should be designed and performed with due consideration of their relevance to human or animal health, the advancement of knowledge, or the good of society.

III. The animals selected for a procedure should be of an appropriate species and quality and the minimum number required to obtain valid results. Methods such as mathematical models, computer simulation, and in vitro biological systems should be considered.

IV. Proper use of animals, including the avoidance or minimization of discomfort, distress, and pain when consistent with sound scientific practices, is imperative. Unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in other animals.

 V. Procedures with animals that may cause more than momentary or slight pain or distress should be performed with appropriate sedation, analgesia, or anesthesia. Surgical or other painful procedures should not be performed on anesthetized animals paralyzed by chemical agents.

VI. Animals that would otherwise suffer severe or chronic pain or distress that cannot be relieved should be painlessly killed at the end of the procedure or, if appropriate, during the procedure.

VII. The living conditions of animals should be appropriate for their species and contribute to their health and comfort. Normally, the housing, feeding, and care of all animals used for biomedical purposes must be directed by a veterinarian or other scientist trained and experienced in the proper care, handling, and use of the species being maintained or studied. In any case, veterinary care shall be provided as indicated.

VIII. Investigators and other personnel shall be appropriately qualified and experienced for conducting procedures on living animals. Adequate arrangements shall be made for their in-service training, including the proper and humane care and use of laboratory animals.

IX. Where exceptions are required in relation to the provisions of these principles, the decisions should not rest with the investigators directly concerned but should be made, with due regard to Principle II, by an appropriate review group such as an institutional animal care and use committee. Such exceptions should not be made solely for the purposes of teaching or demonstration.

Source: Office of Laboratory Animal Welfare, *Public Health Service Policy on Humane Care and Use of Animals*, Office of Extramural Research, National Institutes of Health, Bethesda, MD, 2002.

State and Local Regulations

In addition to the federal regulations governing animal welfare and research facility operations, one must also be aware of the variety of state and local statutes that must be followed. State and local laws can in some cases be more stringent than the federal laws. A facility must follow federal regulations, but if the state and local regulations have requirements that exceed the federal requirements, a facility must also comply with these laws. Many of the state and local laws make an attempt to exempt research facilities from their oversight such as the Pennsylvania dog law. Pennsylvania passed a revised version of their dog law in 2008 that expanded the sections on the requirements to house dogs in a kennel. The housing requirements were directed at improving the conditions of dogs maintained in "puppy mill"–type operations. Pennsylvania's new kennel housing requirements were not directed at research facilities as it was well known that they were inspected annually by the USDA; the state of Pennsylvania exempted research facilities from further inspections by Pennsylvania dog wardens. However, research facilities in Pennsylvania are required to submit their USDA inspection reports to the state once a year. Some states require specific permits to house exotic animals such as NHPs. Therefore, research facilities in these states are required to obtain permits from the state to possess and house such animals. Some states have laws pertaining to the use of animals in consumer product safety testing. New Jersey has a state law that requires alternatives to be used, if they are available. In addition to the Drug Enforcement Agency requirements of obtaining, handling, and record keeping of controlled substances, there are additional state and possibly local laws regarding controlled substances. State and local controls of veterinary practices also exist. In some municipalities in California, declawing of cats is prohibited. Many states have laws against ventriculocordectomy in dogs. States also have "sunshine" laws that require that documents and records at public institutions such as universities to be made available to the public. These laws can lead to public requests of research records from academic institutions. These requests have sometimes led to court proceedings to determine exactly what type of information can be considered proprietary and not open to the public. Disclosure laws are something to consider when performing research or testing at academic institutions. These are just a few examples of the myriad of state and local laws that can and do affect the care and use of animals in research facilities as well as the actual research and testing that is being conducted. The National Association for Biomedical Research (NABR) (www.nabr.org) is an excellent resource for keeping up to date with state and local legislation (National Association for Biomedical Research [NABR], n.d.). In addition to the NABR, state veterinary associations are also good sources of state laws and regulations affecting the local practice of veterinary medicine that may in turn influence the legality of procedures performed on laboratory animals (Pennsylvania Veterinary Medical Association, n.d.).

INTERNATIONAL LAWS AND REGULATIONS

European Union Laws and Regulations

The EU issues directives that members of the union are required to follow. This is the minimum that constituents of the union are expected to meet. If they choose, they can go above and beyond these directives. The new directive, *Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes* (The European Parliament and the Council of the European Union, 2010), covers the requirements that member countries must meet when using animals in research. Organizations or businesses that use animals for research and either are based in European countries or have branch facilities in Europe often require research facilities in non-European countries to meet these directives. One of the main differences between the EU directive and the U.S. requirements is the housing requirements for

dogs and NHPs. The requirements of the "guide" will be used for comparison of housing requirements in the United States. The following are the floor space requirements for dogs: Dogs <15 kg require 0.74 m^2 of floor space, dogs up to 30 kg require 1.2 m^2, and dogs over 30 kg require greater than 2.4 m^2 of floor space. The EU directive requires a minimum of 4 m^2 for dogs up to 20 kg and 8 m^2 for dogs over 20 kg. The EU floor space requirements are approximately four times the floor space required in the United States. The EU minimum floor space requirements can accommodate two dogs without additional space, and every additional dog added to the pen is required to have an additional 2–4 m^2 depending on the weight of the dog. Dogs must be socially housed and can only be separated from each other for a period of time to not exceed more than 4 hours a day. The EU floor space requirements effectively will reduce the number of animals that can typically be housed in a room especially in facilities that were designed when floor space requirements for dogs were significantly less and used cages rather than runs. Studies that may have been accommodated in two rooms essentially would need to be housed in four rooms according to this directive, which will significantly raise the cost of animal care. These costs will then be passed on to clients. In addition to dogs, NHP EU space requirements significantly exceed U.S. requirements. Both the EU and the United States require NHPs to be socially housed. The space requirements for the EU accommodate up to two animals, while the U.S. requirements are based on the individual animal. EU requirements are based on age, while the U.S. requirements are based on weight. In order to perform a direct comparison, "cynomolgus" monkeys will be used as the model. Typically, a juvenile "cynomolgus" monkey used in a toxicology study is approximately 2 years of age and weighs less than 3 kg. For an animal of this size and age, EU space requirements are 2.0 m^2 of floor space and 1.8 m of vertical space. Space requirements for this same animal in the United States would be 0.28 m^2 of floor space and 76.2 cm of vertical space. Two animals would require 0.56 m^2 of floor space in the United States. As with dogs, the EU requirements are approximately 4 times the U.S. requirements. Individual countries in the EU also have some additional requirements, which may be requested when studies are being placed by companies that are based in a particular country.

International Regulations and Resources

A listing of animal research regulations from countries around the world can be found at International Links (International Regulations and Resources) on the website of Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) www.aaalac. org, under Resources. This information has been compiled by AAALAC and questions can be directed to this organization.

Institutional Animal Care and Use Committee Requirements and Function

The AWA came into existence in 1966 to provide guidance and regulations for the care and use of animals used in research, testing, teaching, exhibition, and transportation. The original AWA did not require research facilities to form an IACUC. It was not until 1971 that the NIH, which is a major component of the PHS of the Department of Health and Human Services, issued its first "Policy, Care, and Treatment of Laboratory Animals" reference with which research institutions that were about to receive or were receiving NIH grant money had to comply (Gordon, 1999). This directive first introduced the animal care committee as a means to ensure the proper care and use of laboratory animals. In 1985, the AWA was amended to include the requirement that research facilities institute an IACUC. The current difference between IACUC requirements for the AWA vs. OLAW (which oversees NIH grant monies) is the number of members that are required to be on the committee. The AWA requires a minimum of three committee members: an AV, a chairperson, and an unaffiliated community member. OLAW requires a minimum of five members (AV, chair, unaffiliated community member, nonscientist, and scientist). Members of the committee are appointed

by the chief executive officer (CEO) or highest ranking management individual within an institution or business. The committee reports to the IO. The IO is the person who has the financial authority and resources to assure compliance with the requirements of the AWA and OLAW (United States Department of Health and Human Services, 1988). The responsibility of the IACUC chairperson is to conduct the meetings and report any issues to the IO. The AV must either be specifically certified or possess training and experience in laboratory animal science and medicine or be highly experienced with the use of the species at the institution and must have specifically direct or delegated animal care and use program responsibility. The unaffiliated community member represents the unbiased interests of the general community. The community member cannot be in anyway affiliated with the institution and not a member of the immediate family of a person who is affiliated with the institution. It is imperative that the community member regularly attend meetings, must be made aware of all committee-related activities that occur outside a regularly convened meeting, and is included in the conduct of facility inspections. Unaffiliated committee members can receive compensation for their meeting attendance but not at a rate that is considered to be adding substantially to their regular income. The two additional members required by OLAW, the scientist and nonscientist, are added to help provide a well-rounded membership to participate in discussions. The scientist must be experienced in research using animals and the nonscientist adds a different level of understanding and perspective to the committee from a nonresearch perspective. Typically, the unaffiliated member and the nonscientist are often the ones whose questions in an attempt to understand the rationale for the research/testing being performed lead to a more in-depth evaluation of the procedures being carried out on the animals that may in turn lead to protocol modifications that benefit the animals and the research. OLAW will allow some members to fit into more than one category such as the unaffiliated member can also be considered the nonscientific member as long as he or she is not a scientist. OLAW also allows for alternate members of the committee. Alternates are appointed to the committee by the CEO or equivalent, and each alternate has a one-on-one designation to the category of the voting committee member they can replace (Office of Extramural Research [OER] Web Site, n.d.). They must receive the same training as regular committee members, and they can only vote if the regular committee member they are representing is not present. The AWA does not address the concept of alternate members but does not appear to object to the concept. Neither the AWA nor OLAW limits the number of members that may be on an IACUC committee. It is generally recommended that the number of members on a committee be an odd number to obviate the development of an impasse when voting. Other individuals that may add valuable expertise to the functioning of the committee are animal care staff, statisticians, information specialists, research technicians, etc. (Applied Research Ethics National Association, 2002). One stipulation set by the AWA is that not more than three members of the IACUC may report to the same administrative unit within an organization, business, or institution.

There are a few conflicts of interest that are best to avoid. One is designating the AV as the chair and the other is appointing the IO as a voting member of the IACUC. The AV's role on the IACUC is one of oversight and guidance concerning animal use and welfare. If the AV is also the chair, this can be seen as basically shifting the balance of power to one person. The IO should not be a voting member of the IACUC since the committee reports to the IO, and allowing the IO to vote on IACUC issues could be construed as overly influential. Some recommend that the IO attend IACUC meetings in order to have a better understanding of the function and deliberations of the committee. The benefits of having the IO attend meetings will need to be weighed against the impact of potential influence their presence could potentially have on the committee members and their behavior, depending on the dynamics of the relationship between the IO and the committee.

Responsibilities of the IACUC are delineated in the law (AWA amendments of 1985) and federal policy and regulations (OLAW). The mandate of the committee is to "maintain oversight of the facilities animal care program." This is achieved by the IACUC overseeing the facility's animal care and use program, facility inspections, and protocol reviews. The IACUC must review the

institution's animal care and use program at least once every 6 months. This review provides an ongoing mechanism for ensuring that the institution maintains continued compliance with applicable animal care and use policies, guidelines, and laws. The review also serves as an opportunity for constructive interaction and education for the animal care staff, research staff, and IACUC members. The reviews also aid facilities in preparing for outside inspections and site visits. Key aspects of the review include IACUC membership adequacy, functions, and procedures of the IACUC, how protocols are reviewed by the IACUC, the facility inspection process, provisions for reviewing and investigating concerns regarding animal care and use, record-keeping practices, methods employed to meet reporting requirements, occupational health and safety programs (OH&Ss), veterinary medical program, personnel qualifications and training, and review of written institutional policies such as SOPs. The IACUC may use subcommittees comprised of a minimum of two members for required activities except for protocol review. All members need to be aware of evaluations being performed by subcommittees so they have the opportunity to participate and contribute, if they so desire.

Methods an IACUC may use when reviewing protocols include full committee review and designated member review (DMR). When a protocol is submitted to the IACUC, the following information must be included in the protocol before the review process can begin. There must be a sound rationale and purpose of the proposed use of the animals in a study. The species and number of animals that are to be used must also be thoroughly justified. Housing and husbandry details should be included especially if these conditions differ from routine or recommended procedures. For example, the *Guide for the Care and Use of Laboratory Animals*, 8th edn. (guide), recommends social species be group housed and rodents be housed on solid-bottom caging. If animals are single housed or housed on wire-bottom caging, there should be scientific justification as to why these animals are to be maintained and housed in this manner. The protocol must include a complete description of the proposed use of the animal including the procedures to be performed as well as any drugs that may be used for relief from pain and distress or reasons for disallowance of pain-relieving medications (Committee on Pain and Distress in Laboratory Animals, 1992). If surgical manipulation of the animals is proposed, then preoperative, intraoperative, and postoperative care, including the use of anesthetics, analgesics, tranquilizers, and any other medications, must be adequately addressed in detail in the protocol. Anytime multiple major survival surgeries are proposed, scientific justification must be given. Methods of and reasons for euthanasia must be indicated in the proposal. Humane endpoints must be well defined with criteria and a process for timely intervention, treatment of the affected animals, removal of animals from study, or euthanasia if painful or stressful outcomes are anticipated. The principal investigator (PI) must document consideration of alternatives to painful procedures and provide a written narrative description of the methods and sources used to determine that alternatives were not available. Assurance that the protocol is not unnecessarily duplicative must be provided. Any deviations from the AWA regulation or the guide if funded by the NIH must be identified and approved by the IACUC. The IACUC must be assured that the personnel conducting the procedures on the animals have been adequately trained and are qualified in the procedures being performed.

Full committee review requires that the protocol be reviewed at a convened IACUC meeting with at least a quorum of the members present. The protocol is presented to the committee members by either the PI or a committee member who preferably has expertise or familiarity with the protocol design being submitted. It is advantageous to allow each committee member to have access to a copy of the protocol prior to convening the IACUC meeting. This will allow the members to review the protocol ahead of time and formulate any questions they may have, allowing the meeting to run more efficiently. All deliberations on protocols presented at a full committee meeting must be included in the IACUC minutes. If the PI is a voting member of the IACUC, he or she must abstain from voting on the proposal and is not considered to be a part of the quorum required for deliberations on the proposal. This abstention from voting must be noted in the minutes. When all of this

is complete, then and only then can the committee vote to approve the protocol, require protocol modifications to secure approval, withhold protocol approval, or defer or table the review for additional future discussions (rare).

A DMR process can also be used for protocol review. Each member of the committee is given the opportunity to review all protocols or significant protocol changes prior to review. A member can then call for a full committee review if they deem this is necessary prior to DMR review. If no member calls for a full committee review, then the protocol can go for DMR review. The designated reviewer is then designated by the chair. The designated reviewer can approve the protocol or require modifications in order to secure approval or call for a full committee review. The designated reviewer cannot withhold approval.

Any significant changes to a protocol must be approved by the IACUC before the change occurs. Significant changes include but are not limited to changes in the objective of the study, altering the proposal to switch from nonsurvival to survival surgery, changes in the invasiveness of the procedure, changes in key personnel, changes in the use of pain-alleviating drugs or the nonuse of these drugs, and changes in the methods of euthanasia.

Protocol review is required on a regular basis and the frequency depends on the species being utilized in the protocol that dictates under which regulatory body the protocol falls. If the species being used in the protocol falls under the purview of the USDA, a yearly IACUC review of the protocol is required, and a search for alternatives for protocols containing painful or distressful procedures will be required every 3 years (United States Department of Agriculture, 2011). Protocols that fall under the auspices of OLAW and do not involve USDA-covered species must be reviewed by IACUC every 3 years. In addition to protocol review, postapproval monitoring (PAM) of the approved protocol-driven procedures is highly recommended (Silverman et al., 2007). PAM involves ensuring that the objectives and the procedures stated in the protocol are being followed. PAM can be achieved in many ways such as during the facility inspections that occur every 6 months, quality assurance oversight of protocol activities, veterinary staff rounds, and compliance officer inspections.

Facility inspections involve the IACUC assessment of areas that house animals and their support areas. If a subcommittee is being used to evaluate the facilities, it is highly recommended that the unaffiliated member be a part of the facility inspection subcommittee. Satellite facilities where animals are housed for more than 24 hours must also be inspected. Areas where surgical manipulations are performed are evaluated. The AWA/AWR requires that surgery on nonrodent species be performed in a dedicated surgical suite and surgery on rodent species be carried out in an area that is dedicated for surgery when the surgical procedure(s) is being performed. In accordance to the AWR, animal study areas that hold USDA-covered species for more than 12 hours and holding facilities also need to be assessed. Laboratories where routine work such as weighing, blood collection, and dosing is performed are not required to be evaluated on the inspection, but many IACUCs evaluate these areas on a rotating basis since animal manipulations are occurring in these areas, even though the animals are not actually housed in the area for any length of time. The IACUC during the course of their inspections needs to evaluate caging, physical plant, sanitation, food and water provisions, animal identification, animal health records, controlled drugs, expired drugs, environmental controls, lighting, watering system, occupational and health concerns, staff training, security, knowledge of applicable rules (are employees wearing appropriate personal protective equipment [PPE]), disaster plan, etc. A written report of the semiannual facility inspection must be written and signed by a simple majority of the IACUC members. All deficiencies must be categorized or classified as being either significant or minor. A significant deficiency is defined as a situation that is or may be a threat to animal health or safety. Once any deficiencies are identified, a reasonable and specific plan as well as a time schedule with dates for completion of each finding must be documented. If any significant deficiencies are not remedied by the assigned completion date, the IO must inform the USDA APHIS within 15 days of the lapsed deadline. The report must be sent to the IO in a timely

fashion (usually within 1 month of the completion of the inspection) for review and kept on file a minimum of 3 years (Applied Research Ethics National Association, 2002).

The IACUC must have provisions for reviewing any concerns pertaining to animal welfare raised by the public or institutional employees. Procedures must be established to ensure that any concerns are effectively communicated to the IACUC. The AWR provides personnel with a "whistle-blower" policy. It states that "no facility employee, committee member, or laboratory personnel shall be discriminated against or be subject to any reprisal for reporting violations of any regulation or standard under the Act" (United States Department of Agriculture, 2002). The IACUC chair is typically responsible for ensuring that concerns are investigated but he or she can delegate a subcommittee to handle the conduct of any investigation. Conditions that reportedly jeopardize the health and well-being of the animals must be investigated immediately. In some cases, the veterinarian or a specifically designated individual can evaluate the situation and stop any procedures that do not comply with institutional policy or are perceived as not in compliance with institutional policy until the IACUC can convene and consider the matter formally. The committee needs to formally acknowledge the complaint, and if an investigation is deemed necessary, the findings must be documented as well as the details of any corrective actions, if the situation warrants such. The AWR and PHS policies authorize the IACUC to suspend any activity after review of the matter at a convened meeting of a quorum of the members. Suspensions must be reviewed with the IO in consultation with the committee. Appropriate corrective action is taken and the IO must report any suspension to OLAW and the USDA depending if the grievances involved an NIH-funded study and/or a USDA-covered species.

If the facility has an OLAW assurance number and/or AAALAC accreditation, any departure from the guide must be documented on the semiannual review. Departures from this course of action must be approved by the IACUC prior to the implementation of the departure, or the departure will be considered to be a noncompliance matter reportable to OLAW and AAALAC.

The IACUC committee responsibilities cover many aspects of the functionality of a facility. This committee must be able to ensure to all governing and accrediting bodies that a facility's animal care and use program meets all the standards set by the various entities. It is imperative that all facets of a facility work closely and in harmony with the IACUC, with the ultimate goal being the attainment of compliance with the governing agencies. A strong, compliant animal care and use program equals healthy and well-conditioned animals that lead to good science and ethical and moral behavior as well as a good image to the public.

Euthanasia

The word euthanasia was derived from a Greek word that literally means "good death." In research and testing, euthanasia is very often the final outcome for the greatest majority of the animals. One of the ultimate goals for most individual toxicology studies is the histopathological evaluation of an all-encompassing tissue list that requires that tissues be harvested from the animals. There have been some strides made where imaging techniques are being used to evaluate target tissues such as tumors over time so that animals do not have to be euthanized at each time point. By using these techniques, fewer animals are required for a given study, which in turn leads to less animal death, but the ultimate fate of even the imaged animals is still euthanasia at the end of a study. Culling of animals especially rats and mice in breeding colonies that do not express or harbor the desired traits or gene is commonly performed. Early termination of animals due to endpoints (tumor size, genetic diseases, toxicity, etc.) being reached is a common scenario. Since death is the ultimate outcome, every attempt must be made to make the event as painless and unstressful as possible. Euthanasia is a procedure that is commonly used in veterinary practice, because the euthanasia of animals usually for end-stage disease is considered a standard procedure in veterinary care. Euthanasia of animals unfortunately to control animal populations is also commonplace. In order to standardize and ensure that the best practices are being used to euthanize animals, the American Veterinary Medical Association (AVMA) has developed

Table 18.3 Agents and Methods of Euthanasia by Species

Species	Acceptable	Conditionally Acceptable
Amphibians	Barbiturates, inhalant anesthetics (in appropriate species), CO_2, CO, tricaine methanesulfonate (TMS, MS 222), benzocaine hydrochloride, double pithing	Penetrating captive bolt, gunshot, stunning and decapitation, decapitation and pithing
Birds	Barbiturates, inhalant anesthetics, CO_2, CO, gunshot (free ranging only)	N_2, Ar, cervical dislocation, decapitation, thoracic compression (small, free ranging only), maceration (chicks, poults, and pipped eggs only)
Cats	Barbiturates, inhalant anesthetics, CO_2, CO, potassium chloride in conjunction with general anesthesia	N_2, Ar
Dogs	Barbiturates, inhalant anesthetics, CO_2, CO, potassium chloride in conjunction with general anesthesia	N_2, Ar, penetrating captive bolt, electrocution
Fish	Barbiturates, inhalant anesthetics (in appropriate species), CO_2, tricaine methanesulfonate (TMS, MS 222), benzocaine hydrochloride, 2-phenoxyethanol	Decapitation and pithing, stunning and decapitation/pithing
NHPs	Barbiturates	Inhalation anesthetics, CO_2, CO, N_2, Ar
Rabbits	Barbiturates, inhalant anesthetics, CO_2, CO, potassium chloride in conjunction with general anesthesia	N_2, Ar, cervical dislocation (<1 kg), decapitation, penetrating captive bolt
Rodents and other small mammals	Barbiturates, inhalant anesthetics, CO_2, CO, potassium chloride in conjunction with general anesthesia, microwave irradiation	Methoxyflurane, ether, N_2, Ar, cervical dislocation (rats <200 g), decapitation
Ruminants	Barbiturates, potassium chloride in conjunction with general anesthesia, penetrating captive bolt	Chloral hydrate (IV, after sedation), gunshot, electrocution
Swine	Barbiturates, CO_2, chloride in conjunction with general anesthesia, penetrating captive bolt	Inhalant anesthetics, CO, chloral hydrate (IV, after sedation), gunshot, electrocution, blow to the head (<3 weeks of age)

guidelines for what is considered acceptable, conditionally acceptable (if used in a research setting requires scientific justification and IACUC approval), and unacceptable euthanasia methods in animals. The document is called the *AVMA Guidelines on Euthanasia*, June 2007 (American Veterinary Medical Association, 2007). This is the latest available edition. The AVMA periodically updates this document as more information becomes available on euthanasia methods.

Table 18.3 indicates the methods of euthanasia that are acceptable or conditionally acceptable for euthanizing commonly used laboratory animal species. For other species, please refer to the AVMA document cited earlier.

The following methods are not acceptable for euthanasia: air embolism, concussive blow to the head (for most species), burning, the administration of chloral hydrate (unacceptable in dogs, cats, and small mammals), the administration of chloroform, the administration of cyanide, decompression, drowning, exsanguination alone, the administration of formalin, and the use of various household products or cleaning agents and various solvents.

Organizations Associated with Laboratory Animal Care and Use

Association for Assessment and Accreditation of Laboratory Animal Care International

"AAALAC International is a private, nonprofit organization that promotes the humane treatment of animals in science through voluntary accreditation and assessment programs" (AAALAC, n.d.b). AAALAC inception began with the ACP that held its first meeting in Chicago, Illinois, in 1950.

Members of ACP recognized that there was a need for standards, certification, and accreditation in the use and care of laboratory animals. Over time, members of ACP realized that the accreditation unit should be an autonomous entity and not a part of any particular professional or scientific organization, in order to gain acceptance by the scientific community in general. The founding member organizations of AAALAC numbered 15 and in 1965 AAALAC became an official not-for-profit organization based in Illinois (Miller and Clark, 1999). AAALAC accredits programs both domestically and internationally. Currently, AAALAC has more than 850 companies, universities, government agencies, and other research institutions in 36 countries that have achieved accreditation (AAALAC, n.d.b). AAALAC accreditation for animal care and use programs is achieved by contacting AAALAC and filling out a program description form. This form requires an intensive review of an institution's animal care and use program and includes all aspects of the program such as occupational health and safety, IACUC functions, animal facilities, veterinary care, and SOPs. AAALAC has established a council on accreditation, whose members are responsible for reviewing program descriptions.

The council bases its evaluation of programs primarily on three documents: the *Guide for the Care and Use of Laboratory Animals* (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011), the *Guide for the Care and Use of Agricultural Animals in Research and Testing* (Federation of Animal Science Societies, 2010), and the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes*, Council of Europe (ETS 123, 1986) (AAALAC, n.d.a). AAALAC offers a program status evaluation service to programs that are applying for AAALAC accreditation for the first time. This is a peer-reviewed assessment that allows the institution to determine if its animal care and use program meets AAALAC standards and if not how improvements can be made so that the organization can meet these standards. It also familiarizes the institution with the accreditation process. The advantage of obtaining AAALAC accreditation is that it portrays to the scientific and business communities that a facility is committed to excellence in their humane care and use of research animals (AAALAC, n.d.c). This further indicates that the facility is dedicated to providing a high-quality product in all other aspects of its operations such as preclinical testing and research results.

American Association for Laboratory Animal Science

The AALAS is a membership organization of professionals from around the world who are dedicated to the humane care and use of laboratory animals (American Association for Laboratory Animal Science, n.d.a). AALAS is the premier organization for education in proper laboratory animal care and use. The organization offers programs for laboratory animal technician (LAT) certification at three different levels: assistant LAT, LAT, and laboratory animal technologist. Technicians achieving these certifications indicate a competence in technical knowledge that is nationally recognized. AALAS also publishes two peer-reviewed journals: *Comparative Medicine* and the *Journal of the American Association of Laboratory Animal Science*. These journals contain numerous articles on various topics related to laboratory animals and their use as experimental models, laboratory animal medicine, biology, husbandry, comparative medicine, etc. Other literatures provided by AALAS include the Laboratory Animal Science Professional, Tech Talk, and AALAS in Action. AALAS provides the greatest amount of training resources and continuing education in the industry. Continuing education is provided through the National AALAS Meeting as well as regional and branch meetings. Regional and branch groups provide member networking and support at a local level. AALAS provides a learning library that is used by many training programs around the world. Laboratory animal managers can take advantage of the educational opportunities provided by the Institute for Laboratory Animal Management. Professional and technical awards of excellence are granted each year at the National AALAS Meeting. Job search resources are also provided by AALAS. The AALAS Foundation provides funding and support for programs and materials for public outreach and education on the value of biomedical research.

American College of Laboratory Animal Medicine

The ACLAM is a specialty group founded in 1957 and recognized by the AVMA that provides for the board certification of veterinarians working in the field of laboratory animal medicine (Middleton, 1999). In order to become board certified, a veterinarian must have graduated from an AVMA-approved or AVMA-accredited school or have a foreign equivalency certificate or be qualified to practice in some state, province, and territory in the possession by the United States, Canada, or other countries. The veterinarian must then meet the educational and experience requirements prior to sitting for the board examination. The training required prior to sitting for the board examination currently can be achieved by two methods: through a specific training program in laboratory animal medicine that is of at least 2 years of duration and through 6 years of full-time experience in the field of laboratory animal medicine. The veterinarian seeking board certification must also publish at least a single paper on a relevant topic in a peer-reviewed journal accepted by ACLAM. In addition to the board certification of veterinarians, ACLAM provides continuing education to veterinarians in the field, publishes laboratory animal textbooks, and issues position statements on issues of importance in the laboratory animal medicine field that are used by other organizations and facilities for the advancement of the their animal care and use programs. ACLAM also sponsors research projects that advance the scientific knowledge of laboratory animal medicine and surgery.

Other Associations and Alternative Organizations

There are many organizations (Table 18.4) dedicated to the care and use of laboratory animals as well as those that are seeking for alternatives to animal testing (Table 18.5) (American Association for Laboratory Animal Science, n.d.b). These tables are shown as follows.

Table 18.4   Associations for the Care and Use of Laboratory Animals

ACLAD—American Committee on Laboratory Animal Diseases
ASLAP—American Society of Laboratory Animal Practitioners
AMP—Americans for Medical Progress
ASM—American Society of Mammalogists
ASR—Academy of Surgical Research
AWI—Animal Welfare Institute
ANZCCART—Australian and New Zealand Council for the Care of Animals in Research and Teaching
ARENA—Applied Research Ethics National Association (PRIM&R's website)
CAAT—Center for Alternatives to Animal Testing, the Johns Hopkins University
CALAS—Canadian Association for Laboratory Animal Science
FBR—Foundation for Biomedical Research
FELASA—Federation of European Laboratory Animal Science Associations
FRAME—Fund for the Replacement of Animals in Medical Experiments
ICLAS—International Council for Laboratory Animal Science
ILAR—Institute for Laboratory Animal Resources
LAMA—Laboratory Animal Management Association
LASA—Laboratory Animal Science Association (United Kingdom)
LAWTE—Laboratory Animal Welfare Training Exchange
NABR—National Association for Biomedical Research
NCA—Netherlands Centre Alternatives to Animal Use
PRIM&R—Public Responsibility in Medicine and Research
Scand-LAS—Scandinavian Society for Laboratory Animal Science
SCAW—Scientists Center for Animal Welfare
UFAW—Universities Federation for Animal Welfare

Table 18.5   Alternatives to Animal Testing

Alternatives search service—UCDavis Center for Animal Alternatives.

Alternatives to Skin Irritation Testing in Animals.

ALTWEB—Center for Alternatives to Animal Testing, the Johns Hopkins University.

AWIC—Animal Welfare Information Center.

Center for Animal Welfare, University of California–Davis.

Centre for the Study of Animal Welfare, University of Guelph.

ECVAM—European Centre for the Validation of Alternative Methods.

In vivo imaging community—resources for in vivo imaging researchers.

Information on alternatives databases—hosted by the Norwegian Reference Centre for Laboratory Animal Science and Alternatives.

InterNICHE Alternatives Loan Systems (based in Europe). List of training media and devices.

isogenic.info—having two subwebs—15 steps in the design and statistical analysis of animal experiments and information about isogenic strains.

Model organisms for biomedical research—mammalian and nonmammalian; funding opportunities; NIH.

National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs)—NC3Rs provides a U.K. focus for the promotion, development, and implementation of the 3 Rs in animal research and testing. NC3Rs brings together stakeholders in the 3 Rs in academia, industry, government, and animal welfare organizations to facilitate the exchange of information and ideas and the translation of research findings into practice that will benefit both animals and science.

Netherlands Centre Alternatives to Animal Use—coordinating research and disseminating information on alternatives to animal experiments for the Netherlands.

Norwegian Inventory of Alternatives (NORINA)—a comprehensive collection of information on audiovisual aids and other alternatives to the use of animals in teaching, at all levels from junior school to university.

University of California Center for Animal Alternatives.

Agencies That Provide Regulations and Guidelines on Animal Testing

The safety testing of products in animals such as pharmaceuticals, chemicals, pesticides, and cosmetics is required by law in many countries. The methods of how these tests are to be carried out are dictated by a number of regulatory bodies such as the Environmental Protection Agency (EPA), FDA, Department of Transportation, Pharmaceutical Administration and Regulations in Japan, Organization for Economic Co-operation and Development (OECD), International Committee on Harmonization, European Commission Enterprise Directorate-General Pharmaceuticals and Cosmetics, and European Regulatory Commission on the Registration, Evaluation, Authorisation and Restriction of Chemical Substances. The guidelines that many of these organizations provide and their regulations are in a constant state of flux, and in order to ensure compliance with the various agencies, these regulations and guidelines should be frequently consulted. When referring to agency guidelines, attention must be paid to whether the guideline or alteration of a guideline has been accepted or is merely in draft form. For example, the OECD has a number of changes in their guidelines that are still in draft form and therefore are not the guidance that has been approved and accepted. Many of the changes in the regulations and guidelines address the use of animals. The European directive on cosmetic testing eliminates all animal testing of cosmetics. The testing ban on finished products has been in effect since September 11, 2004, and on ingredients or a combination of ingredients since March 11, 2009 (Commission of the European Communities, 2004). The marketing ban for cosmetics tested on animals for all human health effects has been in force since March 11, 2009, with the exception of repeated-dose toxicity studies, reproductive studies, and toxicokinetics. The marketing ban will apply as soon as alternatives to the three studies listed are validated and adopted by EU legislation with a maximum of 10 years after entry into force of the directive. Once 10 years has past, the marketing ban will apply whether there are validated alternatives or not. The impetus of keeping up with this regulation is that if cosmetic companies use animal

testing and attempt to market their products in Europe, they will be unable to put their product on the market. This can lead to a reduction of profitability for the company. IACUCs must be well versed in the changes in the guidelines and regulations to make sure that when searching for alternatives to painful or distressful procedures, they are aware of acceptable alternatives that are allowed by the various regulatory agencies. These in vitro alternatives need to be seriously considered by the client. An example of an accepted alternative is the local lymph node assay for sensitization where a lower species, the mouse, replaces a higher species, the guinea pig. This assay also reduces the number of animals used on a given study. Organizations such as the Interagency Coordinating Committee on the Validation of Alternatives and the European Centre for the Validation of Alternative Methods (ECVAM) are working diligently to validate alternative testing methods that use less animals and lower-level species and ultimately do not even use animals. Both of these agencies also present their data to the regulatory agencies in order to obtain their acceptance of the alternative test methods.

The FDA has taken the position that they no longer require the Draize testing (acute ocular or dermal irritation studies) or the performance of LD50 determinations. They also want species justification to ensure that the proper species is being used in the safety evaluation of a compound (Schofield, 2011). The FDA has indicated that the NHP should only be used if absolutely necessary, which will require that a suitable justification for their use be provided. In addition to the efficacy of a compound in a particular species, the availability and the feasibility of performing the required techniques in a particular species also need to be taken into consideration. For example, the daily intravenous administration or blood withdrawal in guinea pigs is not feasible unless cannulated animals are used. Once animals are modified, cost as well as the development of complications induced by these modifications must be taken into consideration when planning a study and the interpretation of results. The OECD has modified the LD50 for chemical testing to an up–down procedure that uses fewer animals but still provides the necessary data. In the past, animals had to die on an LD50 study, but now the OECD has established endpoints that may be used to determine if an animal is moribund in order to humanely euthanize rather than to allow it to die on its own (Organization for Economic Co-operation and Development, 2000). Again, one needs to stay abreast of the changes in the guidelines and regulatory requirements in order to ensure compliance with the regulatory agencies and ensure the marketability of a product as well as to use the least amount of the proper species of animals humanely.

Facilities: An Overview

Proper Care and Use of Animals

How a facility that uses animals is managed should be based on the requirements of the animals being housed. The documents that provide guidance on the proper care and use of laboratory animals are the *Guide for the Care and Use of Laboratory Animals* (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011), the *Guide for the Care and Use of Agricultural Animals in Research and Teaching* (Federation of Animal Science Societies, 2010), and the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes*, Council of Europe (ETS 123, 1986), as well as other guidance documents depending on the country of residence or the organizations for which research is being conducted. Various departments of the U.S. government have their own guidelines in addition to the guides (FASS 2012) and the AWA/AWR on the use of animals in research or testing that must also be followed when research or testing is performed or funded by the departments. Examples include the Department of Defense (Department of Defense, 2012), the Department of Veterans Affairs (Department of Veterans Affairs, 2011), and the National Institutes of Mental Health (National Institutes of Mental Health, 2002). In addition to general and agency/department guidelines, other guidelines have been formulated for the care and use of animals used in particular types of research

such as behavioral research (Committee on Guidelines for the Use of Animals in Neuroscience and Behavioral Research, 2003). Recommendations based on species can also be found throughout the literature (Committee on Dogs, 1994). Regulatory bodies such as OECD also have recommendations on the proper care and use of animals for testing that can be found in their testing guidelines. The use of animals in research is a privilege and not a right. To this effect, animals used for research must be treated humanely and with respect as they are living entities with very similar perceptions and reactions to their environment and manipulations that human have. With this in mind, the *U.S. Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training* should be accepted and endorsed (see Table 18.2) by all facilities and organizations that promote and support research and testing such as the Society of Toxicology (Society of Toxicology, 2008). In order to best follow these principles, it is very important to always take into consideration the 3 Rs (replacement, reduction, and refinement) (Russell and Burch, 1959). Whenever a proposal to use animals is made, it should be carefully scrutinized to ensure that the 3 Rs have been applied. Are the proper numbers of animals being used? Through the use of pilot studies and statistical power analysis, the minimal number of animals to use on the study can be determined rather than just picking an arbitrary number of animals that is not based on any type of scientific data or method. Carefully evaluating animal numbers is very important since choosing the number of animals in an attempt to reduce the use of animals may not always be in the best interest of the study and the information needed. Not enough animals may cause the study to fail, which may in turn require the use of more animals as the entire study may have to be repeated rather than conducting the first study with more animals that would allow for proper data collection and interpretation. Reducing animal numbers without good justification can actually lead to an increase in the number of animals used rather than a "reduction." Refinements can often be made to study proposals such as using whole body imaging over time rather than sacrificing animals at each time point. This is not only a refinement but also a reduction in animal numbers. Nursing care for ill animals that can occur with various research models such as nerve injury models and specific disease models is considered a refinement as the discomfort of animals is being alleviated. Complete replacement of animals in testing and research is the ultimate goal when searching for alternatives, but often this is not possible, but it may be possible to use a lower species such as substituting a fish for a rat or rabbit in developmental studies. There are currently a number of assays that have been developed and are being developed to replace the use of animals especially in the field of skin and eye irritation studies that historically have used the rabbit.

Training Personnel

Animal care and research staff training is an important aspect for the success of an animal care and use program as well as the successful collection of scientific data for the benefit of humans and animals. Training can be accomplished through various mechanisms such as hands-on training, professional society meetings with training seminars, online training courses, and in-house seminars. Documentation of training should be implemented, which will allow an accurate assessment of an employee's abilities. Training documentation is also required by many agencies such as FDA, EPA, USDA, and AAALAC. Employee proficiency at a specific task should always be assessed at a timely point prior to the employee actually performing the task in order to ensure that proper animal care and use is being performed. This safeguards the animals as well as the employee from injury and ensures that animals are being taken care of and manipulated in an acceptable manner. The IACUC is also responsible for assuring that personnel working with animals from PIs to animal care staff are properly trained. Proper training is often assessed when PAM is performed by the IACUC. During PAM, the proficiency of investigators and technical staff can be evaluated as study records and actual performance of the technique(s) can be reviewed. The veterinary staff is also responsible for training and determining proficiency of personnel involved in projects, animal

husbandry techniques, handling techniques, dosing techniques, and sample collection techniques. Any deficiencies identified must be immediately addressed and retraining of individuals needs to be implemented immediately. Recertification of staff should also be considered especially for techniques that are not routinely performed and to help curb drift from proper standard procedure. In addition to animal care and research staff training, IACUC members require training to ensure they have the proper knowledge and understanding of their responsibilities as an IACUC member.

Occupational Health and Safety Program

An OH&S should be established by each institution. If the institution is governed by regulatory bodies (USDA, OLAW) or accredited by AAALAC, an OH&S program is required. The OH&S program should ensure that the facility complies with all federal (OSHA), state (DEP), and local regulations and establish safety practices that safeguard employee health. The presence of a safety committee is highly recommended and in some cases required by regulatory agencies to oversee ongoing evaluations of health and safety, conduct facility inspections, facilitate communication, and promote occupational health and safety at the facility. The OH&S program must conduct a risk assessment evaluation of the various procedures carried out at the facility as well as an evaluation of potential exposure to hazardous substances such as chemicals, biologic agents, radiation, physical hazards, and exposure to animals. Based upon a risk assessment, appropriate precautions to guard personnel against potential hazards must be instituted (Committee on Occupational Safety and Health in Research Animal Facilities, 1997). Appropriate precautions include the proper and effective use of PPE. Appropriate PPE must be made available to all personnel working in an area that contains a hazard that requires the use of PPE. Proper PPE will be determined based on the hazards present in a given area. Personnel must receive the proper training on how to use the required PPE, and this training should be documented. SOPs should be written covering when and how PPE should be used. If respirators are part of the required PPE, personnel must receive respirator fit testing yearly as well as periodic medical evaluations to ensure that individuals are physically and physiologically competent to wear a respirator.

Medical evaluations of personnel working with animals should be conducted preemployment and then on a routine basis to assess the development of any physical impairments that could potentially preclude an individual from performing their duties or that may require modifications of an individual's work environment in order to allow them to continue in their current position. An example would be the development of allergies. Latex allergies may require the facility to provide alternate glove material. Animal allergies may require the facility to offer medical treatment for the allergies such as hyposensitization treatment, limitations in the types of species the individual can work with, and/or PPE that provides a higher degree of protection from the presence of airborne allergens. There are cases where some individuals will no longer be able to work with animals and the facility may need to provide them with alternate job requirements. Proper immunization programs should be established as necessary. Personnel working with animals should be current with their tetanus immunization status. If it has been determined that personnel have a risk of rabies exposure such as those working with wildlife, bats and raccoons, with the rabies virus on research projects, rabies prophylactic vaccination should be provided. Personnel working with human-based materials should be provided with the opportunity for hepatitis B immunization. Other immunizations may be offered based on risk assessment. The training and screening for the presence of zoonotic disease should be instituted based on potential exposure to these entities. Personnel working with NHPs should be screened at least yearly for tuberculosis. The measles status of personnel is also important since measles can be contracted from NHPs, or alternatively, personnel with an active measles infection can in turn infect NHPs. Since diseases can be spread from people to animals, policies should be in place to address the proper precautions that should be taken by personnel when they are ill. Policies should also be in place to address personnel with special needs such as

those that are immunocompromised and pregnant. All medical information obtained must meet the confidentiality requirements as well as other medical and legal factors that are required by federal, state, and local laws.

The training of personnel on safety in the workplace is absolutely essential in the animal testing and research environment. Policies and SOPs must be in place to address the proper precautions that must be taken when working in a laboratory setting. Eating, drinking, using tobacco products, applying cosmetics, and handling contacts in laboratories and animal rooms should be strictly forbidden. Proper personnel hygiene should be reviewed such as frequent handwashing, changing into appropriate uniforms and dedicated facility shoes, and showering procedures. Proper facility and equipment safeguards should be in place as appropriate for the hazard being addressed and functioning properly. Policies on facility security should be in place and strictly enforced. Excellent sources of information when developing an OH&S program include the *Occupational Health and Safety in the Care and Use of Laboratory Animals* (Committee on Occupational Safety and Health in Research Animal Facilities, 1997), OSHA regulations, and the *Biosafety in Microbiological and Biomedical Laboratories* (United States Department of Health and Human Services, 2009).

Husbandry

Facilities that utilize animals for research and testing must provide the species housed with the proper environmental conditions, housing, and husbandry requirements. The equipment needed will depend upon the species that is to be housed or the area. It can be as simple as the purchase of filtered polycarbonate shoebox cages on a shelf in an environmentally controlled room to house mice to as complicated as the group housing of NHPs with extensive environmental enrichment devices along with ancillary equipment to separate out the animals when manipulations are required. In the laboratory setting, the temperature, humidity, lighting, and ventilation are controlled to keep the animals healthy as well as to reduce variables in testing and research projects. Parameters for these environmental factors can be found in the *Guide for the Care and Use of Laboratory Animals* (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011). Caging must be adequate for the species that is to be housed and to allow for species-specific behaviors. There are many different types of caging that are available, from wire-bottom or grate-type stainless steel cages to solid-bottom plastic cages. Solid-bottom caging is now being advocated as the standard for the housing of rodents. It is felt that rodents are more comfortable in solid-bottom cages with bedding, and the development and incidence of foot lesions that have been associated with long-term housing on wire-bottom cages are avoided. Wire-bottom cages have been the standard in toxicology testing for many years and still confer the benefits of being able to evaluate the changes in fecal output, fecal consistency, urine amount and color, and the presence or absence of food crumbling. All of these observations can provide a significant amount of information of the effect of a compound on a rodent. Some of these changes may eventually be observed in a rodent on bedding, but the observation is picked up in a more timely fashion when the cage paper under a wire-bottom cage is observed on a daily basis. This can also aid in the more timely assessment of an animal's health since changes in the characteristics of urine and feces can be one of the first signs that an animal may be having health issues. There is some evidence in the literature that rats do not necessarily prefer solid-bottom caging over wire-bottom caging (Rock et al., 2000).

Animals also need to be provided with food and water (generally ad libitum) on a daily basis. Animals must be fed diets that provide them with adequate nutrition, and in some cases where studies are to be conducted under good laboratory practice (GLP), animals are fed certified diets. Certified diets come with a contamination profile so there is evidence that the level of substances considered to be contaminants is below levels that are set by agencies such as EPA and FDA. Diets can also be autoclaved or irradiated to ensure they are sterile when fed to animals that may be immunocompromised or to animals in barrier facilities to prevent contamination of the barrier by

an organism or organisms contained in the diet. As mentioned previously, many species are fed ad libitum, but animals that are housed long term are often limit fed to prevent the development of obesity. All animals must be provided with potable water and this is usually supplied through an automatic watering system or a manual system such as water bottles/sacks and bowls. Water is treated in many ways (reverse osmosis, acidified, autoclaved, etc.) depending on the requirements of the facility and the work they are doing. Cages must be cleaned and sanitized according to a set schedule. How frequently this is done depends on a number of factors. The USDA has specific cleaning and sanitization requirements depending on the species. The "guide" is more opened ended and performance based in the recommended requirements, and the frequency of cleaning is based on "the maintenance of environmental conditions conducive to health and well-being" of the animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011). The "guide" has assumed this stance over time, and with experience and through studying of animal behavior, it has been found that many species do not like to have their caging cleaned too frequently and it is actually stressful to the animals if you change bedding or caging too often. The proper equipment, such as cage washers and tunnel washers, to adequately clean and sanitize caging and accessories must be made available by the facility.

Enrichment

Enrichment programs for laboratory animals should be developed by each facility and approved by the IACUC. The amount and type of enrichment provided will depend upon the research being conducted or the testing with which the animals are involved. Ideally, for GLP studies, enrichment items should have a contaminant evaluation performed on them (certified) and a contaminant profile established, but practically, this may be extremely cost prohibitive as well as severely limiting the types of items that can be used for enrichment. Therefore, at a minimum, items made of inert materials should be used, and for food enrichment, the items should be fit or acceptable for human consumption. Enrichment for various species can run the gamut of caging complexity and type to the manipulation of the variety of different food stuffs. Ideally, items used as toys should be rotated regularly since animals will get bored with the same toys all the time and this does not add to enrichment of the environment. Researchers must also be cognizant of the fact that if certain environmental changes are made, the model being studied may not work as expected since manipulation of the environment can affect the outcome of a study. It has also been found that some types of enrichment can be detrimental to the welfare of the animals causing injury or the development of a guarding type of behavior. When enrichment programs are being planned, research should be performed into the supply of the proper enrichment for the animals by using resources such as the scientific literature, the previous experience of peers, and/or observations recorded from actual in-house studies.

Veterinary Care

Veterinary care must be provided to all laboratory animals. This is a requirement of all agencies and programs that oversee laboratory animal care. The veterinary care program is the responsibility of the AV. The AV ideally should be board certified in laboratory animal science and medicine, but veterinarians with suitable training or experience in laboratory animal science and medicine or those otherwise qualified in the care of the species are also considered adequate for the position. Keep in mind that a nonveterinarian cannot practice veterinary medicine. An adequate veterinary care program consists of the assessment of animal well-being; the effective management of animal procurement and transportation; preventive medicine; understanding of clinical disease, disability, or related health issues; understanding protocol-associated disease, disability, or other sequelae; surgery and perioperative care; and understanding pain and distress, anesthesia, analgesia, and

euthanasia (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011). Trained personnel such as veterinary technicians, animal care technicians, and research technicians can conduct many aspects of the program of veterinary care as long as they remain in timely contact with the veterinarian concerning any issues that may occur. The AV when reviewing protocols must assess the proper use of anesthetics and analgesics provided to the animals in the research proposal. The proper use constitutes selection of the appropriate anesthetic and/or analgesia for the type of research being conducted and species being used (Acred et al., 1994; Baumans et al., 1994). Improper selection can result in the less than adequate control of pain or the confounding or masking of study results. For example, if electroencephalograms are being studied to predict seizure activity, it would be inappropriate to use ketamine for any reason as this can reduce the seizure threshold in animals and actually cause seizure-like activity in animals that normally have a low seizure threshold. The use of ketamine in this situation would confound and/or mask the study results. The frequency of dosing the animals must also be reasonable. If treatment is not frequent enough, breakthrough pain will occur, which does not provide adequate pain control, and too frequent dosing can lead to overdose and the development of adverse consequences such as severe respiratory depression or death as in the case of opioids. The AV should be knowledgeable with regard to the proper selection of analgesics and anesthetics in a wide variety of research situations and have a working knowledge of potential drug and possible procedural interactions as well as provide guidance to the investigators concerning these factors. The AV should also provide guidance to the investigators concerning the performance of the surgical procedures that are being conducted in research proposals. The assessment of proper pre-, intra- and postsurgical techniques such as the use of proper aseptic technique, tissue handling, actual surgical methodology, and appropriate post-op care is the responsibility of the AV. The assessment of the surgical procedure should include a review of the description of the procedure; direct oversight of the actual procedures; review of pre-op, surgical, and post-op records; assessment of animals postoperatively; and review of study clinical observation records.

In addition to surgery and pain management in laboratory animals, the AV also provides guidance on animal procurement, appropriate transportation methods, husbandry, animal handling and restraint, medical treatment, euthanasia, dosing, and sample collection. Animals should be procured from reputable vendors that have been approved by the AV. Animals obtained from approved vendors are least likely to have health problems or infectious disease issues. Following these simple procedures will in turn reduce the times for animal quarantine and avoid or minimize the possibility of an infectious agent from confounding the data. Animals from nonapproved vendors should have extended quarantine time periods, and in some cases, special precautions should be used when handling these animals for the duration of the study in order to prevent the possible spread of any disease or disease agent (cases of subclinical disease) throughout the facility. In the case of animals that are going to remain in the facility for extensive lengths of time, scheduled health screens can be performed at multiple time points, and if infectious disease entities are found, the animals could be rederived. Acclimation times also need to be set to allow the animals to become accustomed to their new environment. Health surveillance programs as well as biosecurity protocols should be in place to ensure animal health as well as the collection of valid scientific data.

The AV should also be providing guidance on multiple aspects of study design and execution of technique. Dose volume limitations and blood volume collection limitations need to be established and are often set based on personal experience and the scientific literature (Diehl et al., 2001; Morton et al., 2001). Vehicle use should be monitored to ensure that the vehicles being used do not cause severe local irritation or systemic toxicity (Gad et al., 2006). Personnel handling the animals and performing various techniques on them need to be properly trained. The AV is responsible for ensuring that personnel are receiving the appropriate training and that the methods are being performed properly. The AV is often involved in helping to develop

new techniques and teaching others to perform these techniques. Animal restraint must be performed properly and be of minimal duration to get the job done. Animals should be acclimated to restraint devices prior to their regular use, and limits should be in place on the length of time that an animal can actually be physically restrained. The appropriateness of chemical restraint should also be considered for animals that pose a safety hazard to personnel and themselves, such as very large animals or NHPs. Appropriate medical treatment should be prescribed by the AV as necessary. The condition of any animal requiring treatment needs to be assessed to determine if the condition is resolving, remaining the same, or getting worse. If the condition is not resolving, the treatment should be changed or the animal should be euthanized. Determination that a condition has resolved and treatment can be stopped should be made by the veterinarian or the veterinarian's designee. The AV is also responsible to ensure that the proper methods of euthanasia are being used and that personnel performing euthanasia are properly trained and proficient at the procedure.

Disaster Preparedness

All facilities should have a disaster preparedness plan in place. A disaster plan is required by USDA, OLAW, and AAALAC. Facilities can experience catastrophic events anytime whether from the forces of nature (i.e., hurricanes, tornados, severe winter storms, earthquakes, pandemics) or from human acts or error (i.e., vandalism, terrorist acts, prolonged power outages, fire). Plans must be in place on how to provide the animals with food, water, heating, cooling, and ventilation when a disaster strikes. While a copy of the plan should be on site, the original version should also be stored off-site. Provisions must also be made if the basic essentials for animals cannot be provided and/or biosecurity has been breached. Responsible investigators should be involved in determining which animals should be relocated if relocation is an option. If animals can be relocated, what animals are going to be relocated and what priority, exactly how they are going to be transported, and to where they are to be transported are all questions that need to be addressed in the disaster plan. If animals cannot be relocated and cannot be provided for in an acceptable fashion during the disaster, they should be humanely euthanized. The possibility of the occurrence of animal injury also needs to be addressed, including the documentation of plans for treatment or euthanasia. The methods of euthanasia and personnel who will perform these methods need to be stated. Emergency contact information must be included in the document and readily and easily available to essential personnel. A chain of command needs to be established so employees know the proper steps to take in the event of a disaster. Personnel safety must be ensured. The disaster plan should be approved by the facility administration or management. It is highly recommended that the disaster preparedness plan be rehearsed by essential personnel to ensure individuals have a working knowledge of their roles in the event of a disaster plan.

Domestic and International Terrorism

AR groups are ubiquitous in countries all over the world, and how they get their message across covers the gamut of expression, from advertisements, media use, and peaceful protests to vandalism, threats, and physical harm. Many of the AR groups outright identify themselves as such and include groups such as the PETA, the Animal Liberation Front (ALF), Stop Animal Exploitation Now, the National Anti-Vivisection Society, and Stop Huntingdon Animal Cruelty (SHAC). Then there are those groups that do not outright identify themselves as AR groups in order to convince the public that they are trying to improve either animal welfare or human welfare. Examples of such covert groups are the HSUS and the PCRM. The goal of AR groups is to abolish the use of animals by humans and even the association of animals in a companion way with humans. The prohibition of the use of animals for research and testing and in education

is one of their primary goals. Different groups attempt to accomplish this in a different way. HSUS tends to use the political arena and the media. This group is large enough and powerful enough, considering their budget is over $100 million dollars a year that it uses its influence to help introduce bills at the local, state, and federal levels. They use their money to help assure that government candidates that support their cause gain offices. Unfortunately, HSUS tends to mislead the public into believing that they are a humane society, meaning they actually use the donations given to them to help animals by caring for them, feeding them, and finding homes for them. They do very little of this and contribute very little money to local humane society shelters. PETA is also attempting to expand their influence by methods other than sensationalism by buying shares in companies such as big pharmaceutical companies so that they can try to influence the shareholder votes concerning the use of animals in research and testing. PETA also uses local ordinances to file petty lawsuits against institutions that use animals for research and testing. They often use state sunshine laws in an attempt to get information on research projects from universities that use animals since universities are publicly funded. They then in turn use this information in sensationalistic and malicious ways to satisfy their own needs—"make money." The interests that these organizations follow are concurrent with the money train. When the money on a given issue dries up, they are off to a new issue. The majority of the AR groups also like to use the media to further their cause. They often create outlandish campaigns to get the media and public's attention. They often take pictures and collect or make statements that are purposely taken out of context to convince the public of the horrible conditions and procedures to which laboratory animals are subjected. Government records such as USDA annual reports and inspection reports are available to the public and are the "golden fleece" for these organizations, since they use these reports and twist the information in them to "show" the public all of the "violations" that are occurring at research facilities and that should incense caring individuals. However, they rarely, if ever, give the complete picture. The methods of protest described here are legal and not violent, but there are groups such as ALF and SHAC that have resorted to activities of harassment and violence to further their cause. ALF has openly and unapologetically made public claims to vandalizing institutions and harassing and physically attacking researchers. The members of SHAC relentlessly harassed Huntingdon Life Sciences (HLS) staff and other companies associated with Huntingdon that provided supplies or services in an attempt to shut the company down. In response to violent acts committed by these groups and others who claim their actions were in the best interests of animals, the United States passed the Animal Enterprise Terrorism Act (S.3880, n.d.) on November 27, 2006. This act allows the Department of Justice to apprehend, prosecute, and convict individuals committing acts of animal enterprise terror. Seven members of SHAC dubbed the SHAC 7 were the first individuals convicted of attempting to shut down an animal enterprise company, HLS, through harassment and threats. These individuals are currently serving various jail times. Other countries are currently passing laws to protect institutions that perform animal research, but there are governments that are buying into the AR movement as a result of public influence and infiltration of government officials by AR advocates. The research community needs to continue to educate the public with regard to the necessity of animal research as a benefit to both human and animal health. Organizations such as the NABR, Foundation for Biomedical Research (FBR) (Foundation for Biomedical Research, 2012), and Americans for Medical Progress (AMP) (Americans for Medical Progress, n.d.) are advocates for biomedical research. These organizations can be very helpful and can provide a copious supply of useful information with regard to AR activities and protection against such activities. They are making a concerted effort to educate the general public about the benefits of biomedical research through the use of various types of media such as educational pamphlets, television commercials, educational materials for schools, billboards, magazines, and novels (Americans for Medical Progress, n.d.).

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Necropsy and Gross Pathology

Charles B. Spainhour

CONTENTS

INTRODUCTION

A well-managed necropsy operation provides a system that assures that all protocol-required tissues and gross lesions are observed, recorded, and properly fixed, for possible future histopathology evaluation. In regulated safety studies, a necropsy is performed to determine cause of death and/or to detect induced or coincidental pathologic alterations in tissues. Typically, a complete necropsy includes examination of the external aspects of the body, all orifices—the cranial, thoracic, abdominal, and pelvic cavities and their contents—and the musculature, subcutis, and bone. However, gross observations only in necropsies can also be performed depending upon the type of study, the objective of the study, or the limitations set by the protocol and the sponsor.

Pathology is defined as a branch of medical science that studies the essential nature of disease, with particular attention being paid to the structural and functional changes in the tissues and organs of a living entity that occur as a result of a disease or some sort of insult (Dorland, 1994). Within the field of pathology itself, there are various subsets of the field. Cellular pathology focuses on changes that occur at the cellular level. Clinical pathology deals with hematologic and clinical chemical changes. Comparative pathology performs comparisons of the responses of organs or tissues to different diseases or insults and also how these changes differ between various animal species. Experimental pathology studies responses to artificially induced disease processes. Functional pathology studies changes in organ or tissue function due to morbid tissue or organ changes. Gross pathology looks at and evaluates large, macroscopic changes to organs that are visible to the naked eye. Surgical pathology deals with the evaluation of organs and tissues that are surgically accessible for diagnosis and treatment. Finally, toxicologic pathology represents an integration of the fields of toxicology and pathology. The beauty of toxicologic pathology is that it requires an understanding of all body systems, including such disciplines as pathology, toxicology, biochemistry, physical chemistry, physiology, medicine, and more (Haschek et al., 2002).

Medically, a necropsy is defined as the postmortem examination of a body, including the internal organs and other structures after thorough dissection so as to determine the cause of death, identify the existence of any pathological changes, and the nature of those pathological changes (Dorland, 1994). Sometimes, the necropsy is mistakenly referred to as an autopsy. By convention, the term "necropsy" is the term used for such an examination of the bodies of animals, and the term "autopsy" is limited to the performance of the postmortem examination of humans. One final point that is worth mentioning in passing is that the term "necropsy" is pronounced as "nek'rop-se" and not "knee-cropsy." The procedure is not an examination of knees, although examination of knees is part of the procedure. This may not sound important, but to many clients and peers, the improper pronunciation of technical terminology does not permit a proper and professional representation of oneself.

NECROPSY ROOM

A room or area that is dedicated completely to the performance or necropsies is essential (Cooper, 1994; Mayer, 1995). The area should be well lit, permitting the accurate evaluation of the carcass and its dissected parts. Ceiling lighting fixtures are commonly 2 ft. by 4 ft. containing

434 W lamps or 2 ft. by 2 ft. containing 235 W lamps. Lamp fixtures should have parabolic louvers and may be recessed, surface mounted, or of a pendant type. Again, the most important aspect of lighting is that it be of such a type as to permit correct color presentation and appreciation. A point worth mentioning is that the new T-8 fluorescent lights that replace the old and now obsolete T-12 fluorescent lights at least in the minds of some people alter the perception of the color of various objects. Tables, cabinets, and countertops should all be of high-quality stainless steel. Other materials can be used, but stainless steel is the best due to its durability, ability to be wet frequently, and ability to be cleaned. Stainless steel shelving and cabinets need to be mounted on the walls and underneath tabletops and counters wherever possible for storage. There needs to be multiple double (preferred over single) sinks with running hot and cold water. A supply of distilled water stored in plastic containers with stainless steel bases and adequate lengths of tubing to facilitate access are important. A floor drain is practical and desirable to facilitate cleaning. The stainless steel work tables should be of heights in the range of 30–36 in., and for each planned work station or work area, provide adequate knee room. Countertops are typically approximately 30 in. in depth. The width of work stations will vary with the size of the species that is being subjected to necropsy, but will typically range from 36 in. to 60 in. Freezers for the storage of autoclaved and bagged carcasses need to be present in the necropsy room or in an adjacent room or contiguous area. Seating is typically adjustable single-seat stools with casters to permit easy movement. Back support is preferred for the comfort of the technicians. Proper attention to the seating will prevent the occurrence of work-related injuries. The presence of an approximately 6 ft. wide vented cabinet (Class II, Type B2, 100% exhaust) for the storage of solvents and corrosives is really essential. Other equipment would include but not limited to mobile light stands, trash containers, tissue grinders, utility carts with casters, and a radiographic film view box or computer for viewing digital radiographs. The room itself needs to be held at a negative air pressure so that airborne particulates and biological and chemical odors are contained. Floors should be of a material that can stand up to frequent washing and disinfection such as epoxy. Floor drains are a desirable feature. Electrical outlets should be present at each planned work station at a minimum and need to be of the GFI type and approved for use in wet areas. Formalin is used in copious amounts during necropsy procedures and is now a known carcinogen. Accordingly, plenty of exhaust vents (laminar flow or elephant trunk) need to be made available at all work areas.

NECROPSY TOOLS

The tools to perform a necropsy will vary not only by species but also by the type of dissection that is required (e.g., eye only vs. whole body) and personal preferences (Feinstein and Waggie, 2011). A typical necropsy work station will provide for the technician a sharp knife, scalpel blades and scalpel handle, dissecting (sharp/blunt nosed) scissors, small surgical scissors, forceps (large, small, serrated, and toothed), bone-cutting scissors or pruning shears, syringes (<10 mL) with needles of various sizes, ligature or string or suture material, cutting boards, paper towels, plastic bags of various sizes, squeeze bottles of 10% neutral buffered formaldehyde (NBF), normal saline and 70% ethanol, cotton-tipped applicator sticks, leak proof screw-top tissue containers, test tubes of various sizes for sample collection, container filled with fixative, tissue cards, plastic cassettes appropriately labeled to identify small individual or paired organs, weighing boats and a multi-compartment plastic tray/trays or bucket to hold animal parts, cassettes, and animal identification. Balances with certified calibration weights suitable for the weight ranges necessary to weigh organs removed from the carcasses during necropsy need to be available. These do not need to be at each work station, but must be present. Some additional tools that do not need to be present at each work station, but need to be available, are an electrical drill, which can be used for some bone work.

NECROPSY TEAM

Participants

To successfully complete a necropsy, the following individuals are typically part of the necropsy team: study pathologist, study director, necropsy supervisor, senior pathology technician, pathology technical staff (animal transporters, blood collector/phlebotomist, dissector/prosector, weighing assistant), and quality assurance representative. The study sponsor may or may not be present. The entire team needs to understand and appreciate that according to Title 21 of the Code of Federal Regulations Part 58 the study director is the single point of study control and he or she alone has the overall responsibility for the technical conduct of the study and for the interpretation, analysis, documentation, and reporting of the study results. For any necropsy, the study director should be present and overseeing the procedure. Depending upon the type of study involved, a study pathologist may be present. When a study is being brought down, the activity level in the necropsy laboratory can be intense, with technical staff and even the sponsor asking a lot of questions all at the same time to both the study director and the study pathologist. Despite this beehive of activity, the study director needs to be kept informed as to everything that is going on with regard to all observations and comments, because the study director according to good laboratory practice (GLP) is ultimately the decision-maker when decisions do need to be made. The study director can and should listen to and take the counsel of the study pathologist and other personnel, but in the end it is the study director that has to be comfortable with all decisions.

This relationship between the study director and the study pathologist participating in the necropsy and very possibly the histomorphological assessment is special. While it is true that the study director is in charge of all aspects of the study, the study pathologist plays a very prominent role nonetheless. A study director should of necessity freely seek the input and opinions of the study pathologist. While it is unclear in this current regulatory climate just exactly how much communication there should be between the study director and the study pathologist and the content of this communication, in the author's experience as long as everything is highly documented so that an unambiguous trail can be mapped out from the initial observations to the recording of the data and the writing of the report, all should be well. A few points need to be made with regard to the role and function of a study pathologist in regulated studies. The study pathologist (1) should be allowed input into the design of the study protocol, especially the sections involving necropsy, tissue collection, and histomorphological analysis; (2) should be allowed full access to the test article information, pharmacology data, study protocol, amendments, deviations, in-life data, clinical pathology data, toxicokinetic data, and necropsy findings; (3) the lines of communication between the study director and the study pathologist have to be open, clear, and unfettered at all times; (4) if there is more than one pathologist working on a study, then clear lines of responsibility must be well-defined and clearly described; (5) the study pathologist should be allowed the responsibility for the interpretation of pathology data; (6) should be allowed to review all study tables and data in the report; and (7) will certainly write his or her own pathology report but should be allowed to assist and work with the study director to write the study report.

Responsibilities

The following is a list of key individuals for the performance of necropsies and brief descriptions of their job responsibilities:

Pathologist or necropsy supervisor: This person is responsible for analyzing information given by the sponsor to determine personnel and equipment requirements. This individual coordinates the necropsy setup, the selection of personnel, and the flow of work with the senior technician assigned to

the necropsy. This person records gross observations and conducts a tissue checkoff of all protocol-required tissues and is responsible to see that each cassette per animal has the proper tissue/organ, identification, and the animal's identification number on it. This person is qualified in the dissection of all species. As a general rule, any study of 28 days or greater duration should have a pathologist present at necropsy.

Senior pathology technician: This individual is responsible for obtaining the supplies and equipment to be used at the necropsy, is responsible for the necropsy setup, and assists in the selection of personnel. This person will also assist the pathologist/necropsy supervisor in training personnel and in supervising personnel at the necropsy. Depending upon the protocol, nature of the study, and the type of necropsy, the senior pathology technician may be designated as the overall necropsy supervisor to record gross observations at necropsy and conduct a tissue checkoff of all protocol-required tissues. This person is qualified in the dissection of all species.

Pathology technician: This individual is responsible for assisting the senior pathology technician in necropsy setup and work flow. The pathology technician should be capable of dissecting all species of animals.

Prosector: This person is responsible for dissecting animals, relating all gross observations to the pathologist and/or necropsy supervisor. This individual is also responsible for confirming each animal's identification number and writing that number on each tissue cassette for each animal.

Phlebotomist: This individual is responsible for the collection of blood samples as required by the protocol.

Weighing assistant: This person is responsible for the following tasks: (1) calibration of scales or confirmation that scales have been properly and acceptably calibrated, (2) confirms that tissues to be weighed have been properly trimmed of excess fat and connective tissue, and (3) ensures that tissue weights are recorded properly and informs the pathologist or pathology associate of possible weight deviations from normal and have the deviations verified.

Training

Necropsy teams can either be dedicated (the performance of necropsies is their only job) or comprised suitably trained toxicology technicians, some of whom may have actually worked on the study being brought down. Dedicated necropsy teams can be very efficient in their activities, but their existence adds cost to an organization's operation and they must be kept busy all of the time to be cost effective. Appropriately trained toxicology technicians are more cost effective but may be a bit slower in their throughput. Of course, there can be compromises in the staffing of a necropsy laboratory, such as a permanently assigned necropsy supervisor and senior pathology technician, etc., with other positions being filled as necessary from the toxicology technical pool. There is an unsubstantiated feeling that dedicated necropsy teams are better trained than toxicology technicians performing necropsies. Regardless, personnel selected to perform necropsies needs to have successfully completed a thorough necropsy training program. If the training is executed properly, there should be no difference in the quality of performance between a dedicated necropsy team and appropriately trained toxicology technicians. The training session is typically conducted by a pathologist, the necropsy supervisor, and/or the senior pathology technician. Training sessions generally consist of lectures, videos, and actual necropsy participation. The following topics should be covered in the execution of this program: (1) discussion of GLP as related to the performance of a necropsy, (2) discussion of methods used in the proper handling of tissues and proper terminology used to describe gross observations, (3) discussion of the necropsy participants and necropsy team members' individual responsibilities, (4) dissection techniques, (5) animal identification, (6) methods of sacrifice, (7) tissue identification, (8) lesion observation, and (9) use of the necropsy data collection sheet or necropsy electronic data collection program. If necropsies are subcontracted to an extramural organization, then a decision has

to be made under whose standard operating procedures (SOPs) the necropsy will be performed. Usually in these cases, it is the SOPs of the subcontractors that are followed.

PRENECROPSY MEETING

Prior to the initiation of any necropsy, a prenecropsy meeting with the members of the necropsy team should be held in order to assure the best possible outcome for the activity. The following points should be discussed as required by the study protocol or at the sponsor's request: (1) the nature of any clinical or in-life findings, (2) the list of protocol tissues required to be collected and potential target organs, (3) the record of any clinical pathologic changes, (4) the expected number of animals on which the necropsies to be performed, (5) the animal species, sex, age, and strain, (6) group and animal numbering and identification scheme, (7) what blood samples are required to be collected and details of collection (volume, route, etc.), (8) any special requirements (e.g., organ weights, perfusions, bone marrow or blood smears, photography, electron microscopy sections, etc.), (9) any precautions that need to be taken during the necropsy, and (10) assignment and review of necropsy personnel and their responsibilities. A prenecropsy meeting is not required for studies that require only a gross necropsy.

NECROPSY FORMS

Observations Recording

In preparation for and prior to the performance of any necropsy, a copy of the sponsor's study protocol and a listing of animal numbers (by animal group and sex) from the study need to be obtained and a pathology project number be assigned and recorded in the pathology study logbook. Necropsy gross pathology data can be recorded on individual necropsy sheets or captured into an electronic database. One possible format for a necropsy sheet or electronic database to record gross findings is shown in Figure 19.1. This protocol-specific, species-specific, sex-specific necropsy sheet when generated (paper or electronic) will serve as the official necropsy record for each animal and be referenced for future histopathology evaluation. This form should contain the following:

1. Pathology project number.
2. Study number.
3. Contract or any other identifying number.
4. Species of animal.
5. Animal identification number.
6. Animal group number.
7. Animal sex.
8. Method of sacrifice
9. Fixation method (if all tissues are not fixed in 10% NBF and specify exactly what tissues were preserved in ach particular fixative).
10. Day of study.
11. Animal fate, found dead or moribund sacrifice.
12. Comments (if necessary).
13. Tissue listing or a compilation of tissues and organs to be taken at necropsy. This list will include all tissues and organs to be examined, collected, and saved at necropsy. This list is compiled when the form is prepared prior to the necropsy. Tissues are checked off as they are placed in fixative.

Study Number			Animal Number		
Histo Number			Group		
Pathology Number			Species: Rat	Sex: Male	
Day #			Body Weight (g)		
Moribund Sacrifice			Scale Number		
Found Dead			Fixatives: 10% NBF		
Method of Sacrifice: CO2 Asphyxiation			Bouin's—testes Davidson's—eyes		
Tissue	√/A	Weight (g)	Tissue	√/A	Weight (g)
Identification (verified)		—	Mammary gland		—
Adrenals			Pancreas		—
Aorta		—	Pituitary gland		—
Brain			Prostate		—
Epididymides		—	Nerve—sciatic		—
Esophagus		—	Salivary gland(s)		—
Eye with optic nerve		—	Seminal vesicles		—
Femur with articular surface		—	Skin		—
Heart			Spinal cord	—	—
Intestine—large	—	—	Cervical		—
Cecum		—	Lumbar		—
Colon		—	Thoracic		—
Rectum		—	Spleen		
Intestine—small	—	—	Sternum with bone marrow		—
Duodenum		—	Stomach		—
Jejunum		—	Testes		
Ileum		—	Thymus		—
Kidneys			Thyroid/Parathyroid		—
Lacrimal glands		—	Tongue		—
Larynx		—	Trachea		—
Liver			Urinary bladder		—
Lung with main stem bronchus		—	Gross findings		—
Lymph node	—	—			
Mandibular		—			
Mesenteric		—			
Lesion Description					
Organ					
Prosector/Date:			Comments		
Weigher/Date:					
Pathologist/Supervisor: Date:					
√/A Column code: √ = normal, saved in fixative; A = abnormal, saved in fixative; M = missing; (—) = not applicable.					
Weight column—paired organs weighed together.					

Figure 19.1   Sample necropsy sheet for male rat.

14. √/A column—each line that has an organ or tissue in it should contain a "√/A" block that will house one of the following entries (blanks are not allowed): √ (the organ/tissue is present, appears normal, and has been placed in fixative), "A" (The organ/tissue is present but contains a lesion or deserves a comment and has been placed in fixative. If this entry is used, an entry is required in the "lesions" section of the form.), and "M" (the organ or tissue is missing). For animals that have been cannibalized, a "C" should be used and (–) for not applicable. It should be noted that if a lesion is present in either organ of a paired set, then an "A" is placed in the block following the organ, and the left or right organ containing the lesion is described in the lesions section of the form. If one of the paired organs is not present at necropsy, but the other is present and normal, then an "N" is placed in the box following the organ, and a comment is made in the lesions section (e.g., left adrenal missing).

15. "Lesions"—for each "A" placed in any box, an entry is required in the applicable spaces of the "lesions" section as follows: "organ" (an entry is always required and it should be the same as the organ for which the designation "A" was given) and "description." (Lesion(s) descriptions should be entered in the box immediately next to the organ or tissue listed as having the lesion. This description may include location, color, size, weight, shape, consistency, number or percent of involvement, content, and odor.)

16. Terminal body weights (grams or kilograms).

17. Terminal body weight in grams or kilograms.

18. Organ weights—organs are weighed prior to fixation or postfixation, as required by the protocol. The identification number of the scale needs to be recorded.

19. The person performing the necropsy should initial and date the form.

20. The person weighing the organs should initial and date the form at the completion of the weighing procedure.

21. The supervisor or supervising pathologist of the necropsy will initial the form and date it.

22. Comments. Any relevant observations or information that may be of value in later interpretation of the necropsy should be recorded, initialed, and dated on the form.

When performing gross only necropsies in which only a limited number of animals, organs, or tissues will be evaluated, the recording of gross observations for multiple animals can be accomplished on a single necropsy sheet or on a general observations page of the study notebook.

Documentation and Procedure for Missing Tissues

Previously, we have mentioned missing tissues. Tissues or organs can be missing because of agenesis, maceration, or destruction in the dissection process, or the tissue or organ is just plain lost in the beehive of activity that occurs during the performance of the necropsy. In cases of agenesis and destruction of the tissue or organ, all that one can do is to just completely and thoroughly document the situation. However, when a tissue or organ shows up missing, every effort must be made to try and find the missing tissue or organ. The best way to do this is to stop what you are doing immediately and then meticulously retrace one's steps over a time period that brackets when the potential loss might have occurred. Sometimes, tissues or organs get placed in the wrong compartment of an animal tissue or organ tray. Although it does not sound very glorious, a little bit of "trash can diving" might well be in order. Again not very professional sounding, this is an activity that can and frequently does produce positive results. If the tissue or organ is found, place it in its appropriate place and document. If the tissue or organ is not found, all one can do is just document that it was lost during necropsy. While such a fate is not desirable, it does uncommonly happen. If it is happening too frequently in the performance of necropsies, then the necropsy process might be flawed, the necropsy participants are working too quickly, or the necropsy personnel are not paying attention to the details of their task and need to be counseled. Retraining of personnel may well be in order or a reevaluation of the flow of work during necropsy can be useful in resolving the problem.

Documentation and Procedure for Unscheduled Deaths and Necropsies

We have also made previous reference to unscheduled deaths and unscheduled necropsies. It cannot be emphasized enough that animals that are to be subjected to necropsy should be recently killed in order to avoid the potentially confounding and deleterious effects of autolysis. If this is not possible, then refrigeration of the carcass is the next best option. Never should a carcass be frozen, because freezing and the ultimate thawing will produce significant tissue damage and complicate the histopathology assessment. All this said, animals on studies do not always read the protocol and accordingly sometimes decide to die as a result of test article toxicity on their own schedule and not in concurrence with the termination schedule set forth in the study protocol (Scenario A). Additionally, the technical staff may (rare event) help things along, and an animal must be sacrificed as a result of some sort of handling-induced injury (i.e., gavage error, significant skeletal injury (broken bone), getting crushed between a cage and the cage rack, etc.) (Scenario B). The key point in dealing with any unscheduled death is to try and plan and arrange things so that the necropsy can be performed as close as possible to the time of death, thereby minimizing the contribution of tissue autolysis. For Scenario B, the animal will need to be euthanized, and with proper planning, the necropsy can be performed on a freshly dead carcass. Here, normal necropsy procedures are followed, and any required biological fluids should be collected. In Scenario A, if an animal is just found dead, it should be subjected to necropsy as soon as possible after its discovery using typical normal necropsy techniques as described here. However, due to the presence of autolysis and cell death, the value of any samples collected of any required biological fluids is highly questionable. These fluids can be collected if desired and saved or even analyzed but need to be appropriately marked and annotated in the study record that the samples came from a dead animal and not a live one. Because of its importance, let's repeat the aforementioned referenced point that if any animal that is found dead, if it cannot be subjected to necropsy immediately or as soon as possible, it should be refrigerated and "not frozen." To avoid or lessen the frequency of these types of situations, the astute study director needs to have complete control of his or her study, understand the pharmacology and toxicology of the test article, have educated the technical staff for the potential of unscheduled death, and possibly change the schedule (i.e., frequency, timing, etc.) for performing observations of the animals with special emphasis on the development of any premonitory signs suggesting death. However, due to cost considerations, some clients will not want to pay additional money for more monitoring, which is unfortunate. The organization or staff may also have human resource limitations making enhanced observations not possible. Finally, some animals just peracutely die for a variety of reasons and there is little that can be done about that. Regardless, a competent and experienced pathologist will be of great help and be able to ameliorate this situation as he or she will easily be able to discern the differences in changes in tissue as a result of agonal change, autolysis, or trauma. A subclassification of Scenario A is animals that are found in a moribund state. Moribundity means that a living thing is in a dying state and very near death. It is important to appreciate that the biochemistry and physiology of the animal are not normal. There are two schools of thought with regard to the handling of these types of animals. One position is that no samples of any biological fluids should be collected because of the altered biochemistry and physiology. The alternative view is that the animal is still alive and every effort should be made to collect as much information as possible and accordingly any biological fluid required by the protocol. While it is true that insights may be gained from these samples with regard to a mechanism of death, most likely the data generated from these samples will be aberrant. In reality, unfortunately and far too often, if the data from such samples fit one's hypothesis, then the data points are used, and if they do not fit the hypothesis, then they are not used. This is not good science. Regardless of one's position on this point, if the data from such an animal are used, there needs to be "no" ambiguity in the study record with regard to documentation of the nature of the sample and the state of the animal and

any biological fluid samples collected are not normal samples. Normal necropsy procedures should be followed for harvesting tissues. However, one big difference with scheduled versus unscheduled deaths is that the study pathologist or a pathologist is not present for the necropsy.

TISSUE COLLECTION

Many times, highly detailed procedures regarding the execution of a necropsy will be delineated in the study protocol. However, when procedures are not specified by the study protocol or the sponsor, the following procedures are recommended for the following: (1) Unless animals have been previously randomized for blood sampling, animals are sacrificed in numerical order across the consecutive study groups to minimize the confounding effects of group-to-group variation and intradiurnal physiologic and biochemical changes within animals. (2) The order of removal of tissues should follow a standard necropsy procedure such as described here, but techniques may vary slightly depending on the sponsor's study design, the protocol, or if extensive lesions in one or more organ systems require a specialized and detailed dissection and/or description. Terminal body weights should be collected at an appropriate time from all animals at the end of a study. These weights are very central to the determination of accurate and meaningful organ–body weight ratios. To minimize variation, these weights should be collected at the time of necropsy and not as the weights of the animals the morning of the necropsy. Special considerations for the performance of necropsy may include but not limited to the following: (1) Target organs may be removed first (e.g., in an inhalation study, the lungs should be removed first). (2) Tissues are to be removed with forceps by grasping surrounding, attached, or contiguous membrane to prevent handling artifacts. (3) Tissues may need to be incised in such a fashion as to provide the maximum exposure to the fixative (e.g., liver lobes may be incised to ensure proper fixation; the brain may be fixed in toto). (4) The use of a fixative other than the standard fixative that is 10% NBF.

FIXATION

Fixation is performed to kill the tissue, prevent the development of "postmortem" decay (autolysis), and preserve the biological material in a state that is as close to its natural state as possible (Carson, 1997; Culling et al., 1985; Kiernan, 2000; Preece, 1972). A fixative denatures or destroys biological molecules especially proteolytic enzymes, thereby preventing an internal-based degradation. Fixatives also protect a sample from the decay and damage from microorganisms. Since fixatives chemically alter cells and tissues on a molecular level, their mechanical strength, rigidity, and stability preserve morphology as the tissue sample is processed. Fixation is the first step of a multistep process preparing biological materials for microscopic evaluation. There are multiple types of fixation such as heat fixation, perfusion, and immersion. Immersion is the most commonly used method of fixation, and in this procedure, a tissue is immersed in a fixative of volume that is 15–20 times greater than the volume of the tissue to be fixed. In order to become fixed, the fixative must diffuse through the tissue. So tissue size, density, surface area, thickness, and the type of fixative must be considered. The larger the sample, the longer it takes for the fixative to reach the deeper parts of the tissue and the longer it takes for a material to become fixed, so the higher the level of autolysis that will be found in the sample. The most commonly used fixative in histology is formaldehyde. It is usually used as 10% NBF. It acts as a cross-linker creating covalent chemical bonds between proteins in tissue, anchoring soluble proteins to the cytoskeleton, and providing additional rigidity to the tissue. Other types of chemical fixatives include but are not limited to precipitating fixatives (alcohols), oxidizing agents (potassium dichromate), mercurials (Zenker's fixative), picrates (picrate salts),

Hepes-glutamic acid buffer-mediated Organic solvent Protection Effect (HOPE), and frozen section. The common names of fixatives frequently used in toxicology necropsies are formalin, Zenker's I solution, Karnovsky's fixative, Bouin's solution, Davidson's fixative, phenol red, and 90% isopropyl alcohol. The fixative of choice for proteins is NBF; for enzymes, frozen section; for lipids, frozen section or glutaraldehyde/osmium tetroxide; for nucleic acids, alcohols or HOPE; for mucopolysaccharides, frozen section; and for glycogen alcohols or for biogenic amines, NBF or Bouin's. There are multiple factors that can affect fixation and these include the following:

1. pH, which should be kept within the physiological range of pH 4–9. The pH for ultrastructure preservation should be kept between 7.2 and 7.4.
2. Osmolarity—hypertonic solutions cause cell shrinkage and hypotonic solutions cause cell swelling and poor fixation.
3. Specimen size should be ideally between 1 and 4 mm in thickness.
4. Volume of fixative, which should be 15–20 times the tissue volume.
5. Duration of immersion or fixation should be approximately 1 hour/mm of tissue. One might think that longer periods of exposure might be better, but just as underfixation can lead to the generation of poor quality samples, overfixation can be equally detrimental to the quality of the tissue specimen. So follow the aforementioned recommended guidelines as best as possible.

NECROPSY AND GROSS PATHOLOGY TERMINOLOGY

All of the lesions that are observed during the course of the performance of a necropsy need to be documented in a precise and concise manner (King et al., 1989; Strafuss, 1988). The written description must be able to generate in the mind of the reader a picture identical to that which was observed on the necropsy table. While it sounds simple, for some reason, many individuals seem to have problems with these types of descriptions. Use the things that you are familiar with to help with your written observations, such as mahogany brown in color, cobblestone-appearance surface, lemon yellow color, glistening or shiny, sandpaper like, port-wine red in color, etc. The description of any lesion should incorporate not necessarily all of the following features but those that are "appropriate," such as the location, color, size/weight, shape, consistency, number or percent involved, time, severity, content, odor, or cause. To put it simply, just write what you see, feel, or smell and the description will be perfect. Pathology has its own language, as do many areas in science and medicine. While one does not have to become a pathologist, it is important for reasons of personal presentation and effective communication with a pathologist and sponsor as well as peers that at least a minimal glossary of words and their definitions be understood. Use the terminology correctly. For example, if there is pus present, the lesion or area is not "pussy." If you cannot speak the term in public, then you cannot write it. The proper term is that purulent material is present. Morphological descriptions and interpretations of lesions may include the following:

1. Distribution for an organ or organs—unilateral versus bilateral, focal versus multifocal, and locally extensive versus diffuse.
2. Timing—peracute, acute, subacute, chronic, or chronic active.
3. Severity—minimal, moderate, marked, slight, or severe.
4. Distribution for the whole body—localized versus generalized.
5. Presumptive cause—verminous, toxic, chemical, viral, traumatic, mycotic, or bacterial.
6. Type of lesion or finding—purulent, fibrinous, fibrinopurulent, or hemorrhagic.
7. Appearance of cut surface of the organ or tissue.

The location of lesions should be described using well-defined anatomical reference points. Remember that sizes should be represented as best as possible in three dimensions (linear units of

millimeters or centimeters), volumes in milliliters or liters, and weight in grams. For hollow organs, the amount, appearance, and odor of the contents should be described. Be careful when ascribing a cause and always precede such documentation with the word "presumptive." Remember for GLP studies one does not want to possibly put themselves into a contradictory position at some later time point by making a premature diagnosis or assessment that may later prove to be incorrect. For example, one might be inclined to state that a material is blood because it is red and liquid. The more correct observation would be a "red in color, clotting or nonclotting, viscous or watery fluid, presumptive blood." In cases of paired organs, where only one organ is affected, remember to identify which one is affected. The proper usage of terms takes some practice, but with practice, the terms become commonplace language. Do not be afraid to touch, squeeze, palpate, cut into (produce a cut surface), or put your nose near the carcass to gain a full appreciation of the sample and the moment. This animal died for the sake of medical science, so make the most of the data-gathering opportunity. For a basic and limited compendium of highly relevant terminology, please refer to Tables 19.1 through 19.4.

Table 19.1   Terms for Gross Morphology and Distribution of Findings in Necropsy

Gross Morphology Entries	Qualifiers for Distribution
Accumulation	**Distribution**
Adhesion	Confluent
Alopecia	Diffuse
Amputation	Multiple
Calculus	Number
Crust	Single
Cyst	
Deformity	**Color**
Dilation	Black
Diverticulum	Blue
Emphysema	Brown
Enlarged	Clear
Fluid	Cloudy
Focus	Dark
Foreign body	Green
Fracture	Gray or grey
Hernia	Mottled
Intussusception	Opaque
Laceration	Pale
Lesion	Pink
Mass	Purple
Nodule	Red
Obstruction	Tan
Parasite	White
Perforation	Yellow
Pigmentation	
Prolapse	
Rupture	
Scar	
Small	
Thick	
Thin	

Table 19.2   Necropsy Terminology

Accumulation—A collection of material in a given area (e.g., dried blood, feces, etc.).

Adhesion—Abnormal fibrous union of an organ or part to another.

Alopecia—Loss of hair, may be partial or total.

Amputation—The removal of a limb or other appendage or outgrowth of the body.

Brittle—Liable to break or snap, friable.

Calculus—A solid concretion composed chiefly of mineral substances and salts found in ducts, passages, hollow organs, and cysts.

Caseous—Resembling cheese or curd; cheesy.

Clear—Absence of coloration; transparent.

Cloudy—Obscured, not limpid or clear.

Color—The following may be used; black, blue, brown, green, gray (or grey), pink, purple, red, tan, white, and yellow.

Confluent—Becoming merged; not discrete.

Crust—A bark-like hard covering; especially a dried exudate on the skin.

Cyst—An enclosed space within a tissue or organ, lined by epithelium, and usually filled with fluid or other material.

Dark—Of a deep shade; black or almost black.

Deformity—The state of being misshapen. Marked deviation from the normal in the size or shape of the body or part, congenital absence of a portion, or all of a body part.

Depressed—Sunk below the surface.

Diffuse—Not definitely limited or localized; widely distributed.

Dilation—The condition, as of an orifice or tubular structure, of being dilated or stretched beyond the normal dimensions.

Diverticulum—A circumscribed pouch or sac of variable size occurring normally or created by herniation of the lining mucous membrane through a defect in the muscular coat of a tubular organ.

Emphysema—A pathological accumulation of air tissues or organs applied especially to such a condition of the lungs. An anatomical alteration of the lungs characterized by abnormal enlargement of the air spaces distal to the terminal respiratory bronchiole often accompanied by destructive changes in the alveolar walls.

Enlarged—Measurably larger than normal size.

Fibrinous—Pertaining to or of the nature of fibrin. An elastic filamentous protein.

Firm—Relatively solid, compact, or unyielding to touch.

Flat—Smooth and regular with few or no hollows or depressions.

Fluid—A fluid substance, such as any liquid secretion of the body.

Focus—A small (usually < 5 mm) circumscribed alteration of color or consistency; single site; foci (plural).

Foreign Body—A substance occurring in any organ or tissue where it is not normally found.

Fracture—A break in bone, cartilage, or solid organ usually caused by trauma.

Friable—Easily crumbling or breaking into pieces (often used in reference to livers).

Gelatinous—Resembling gelatin or jelly.

Granular—Composed of, like, or containing grains or granules.

Gritty—Like, containing, or consisting of grit.

Hard—Resisting indentation, incision, or compression; solid; unyielding.

Hernia—Abnormal protrusion of an organ or a part through the containing walls of its cavity; beyond the normal confines.

Intussusception—The prolapse of one part of the intestine into the lumen of an immediately joining part. The receiving of one part within another.

Irregular—Lacking symmetry or uniformity.

Laceration—A tear or wound made by tearing.

(Continued)

Table 19.2 (*Continued*)   Necropsy Terminology

Lesion—An alteration, structural or functional, due to disease. Any pathological or traumatic discontinuity of tissue or loss of function of a part. Very nonspecific. Use more specific designations wherever possible.

Linear—Of or pertaining to a line or lines.

Lobulated—Made up of or divided into lobules.

Mass—A circumscribed enlargement of an organ or tissue. May be irregular in shape. Usually applies to larger (> 1 cm) lesions. See also nodule.

Mottled—Marked with spots or blotches of different color or shades of color.

Multiple—More than one part, aspect, etc.

Nodule—A circumscribed enlargement or solid elevation of varying size. A nodule is relatively smaller than a mass (usually < 1 cm).

Number—Use a whole number.

Obstruction—The state of being occluded or stenosed, applied especially to hollow ducts and vessels; blockage or obstacle.

Oily—Of, pertaining to, containing oil; greasy.

Opaque—Impervious to light; not translucent or transparent; having no luster; dull.

Oval—Having the shape of an egg.

Pale—Of a very light shade of color; lacking in brightness or intensity of color.

Papillary—Pertaining to or resembling a small nipple-shaped projection or elevation.

Parasite—An organism that lives, during all or part of its existence, on or in another organism, its host at whose expense it obtains nourishment.

Perforation—A hole made through a part of the wall of a cavity or tissue surface produced by a variety of means.

Pigmentation—Coloration resulting from any normal or abnormal coloring matter of the body (i.e., bile, hematogenous [derived from blood] or ceroid pigment).

Plaque—Any patch or flat area.

Polypoid—Resembling a polyp (a protruding growth especially from mucous membrane).

Prolapse—The falling or sinking down of a part or organ, especially its appearance at a natural or artificial orifice.

Punctate—Resembling or marked with points or dots.

Raised—Elevated in low relief.

Round—Having a contour that is circular or approximately so.

Rubbery—Having the consistency of a resinous elastic material.

Rupture—A forcible tearing of a part or disruption of tissue; hernia.

Scaly—Scale like; characterized by scales.

Scar—A mark remaining after healing of a wound or other disease process. A permanent mark resulting from a wound or disease process in tissue, especially the skin.

Single—Consisting of one only; individual.

Size—Record dimensions in millimeters.

Small—Measurably less than normal size.

Soft—Less firm than normal.

Spherical—Shaped like a sphere.

Thick—Relatively greater than normal in depth or extent from one surface to the opposite.

Thin—Having a relatively smaller distance than normal between opposite sides or surfaces. Not great in diameter or cross section.

Viscous—Semifluid; sticky.

Gross morphology, distribution, and color

Volume—Record volume of fluids in milliliters

Watery—Resembling water; thin or liquid

Table 19.3   Topographical Terminology (Organ and Site) Used to Locate Gross Lesions

Adrenal Gland	**Meniscus**	**Olfactory Lobe**	**Ear**
Bilateral	Metacarpal	Parenchyma	Bilateral
Capsule	Metatarsal	Pineal gland	Canal
Cortex	Patella	Pons	Internal ear
Left	Pelvis	Right	Left
Medulla	Phalanges	Unilateral	Middle ear
Right	Proximal	Ventricle	Pinna
Unilateral	Radius	**Carcass**	Right
Blood	Rib		Unilateral
	Right	**Cavities**	**Epiglottis**
Blood Vessel	Sacral	Abdominal	
Abdominal	Scapula	Cranial	**Epididymis**
Anterior	Shaft	Mediastinum	Bilateral
Aorta	Sternum	Nasal	Head
Carotid artery	Synovial tissue	Oral	Left
Jugular vein	Tarsal	Pelvic	Right
Mesenteric artery	Tendon	Pericardial	Tail
Posterior	Thoracic	Scrotal	Unilateral
Pulmonary artery	Tibia	Thoracic	**Esophagus**
Thoracic vena cava	Turbinate	**Clitoral Gland**	Lumen
Bone	Ulna	Bilateral	Mucosa
Articular capsule	Unilateral	Left	Wall
Bilateral	Ventral	Right	**Eye**
Bursa	Vertebra	Unilateral	Anterior chamber
Calvarium	**Bone Marrow**	**Coagulating Gland**	Bilateral
Carpal	Calvarium	Bilateral	Conjunctiva
Cervical	Femoral	Left	Cornea
Coccygeal	Humerus	Lumen	Left
Cortex	Mandible	Mucosa	Lens
Cranium	Maxilla	Right	lids
Distal	Sternal	Serosa	Optic nerve
Dorsal	Thoracic	Unilateral	Retrobulbar
Femur	Tibia	Wall	Right
Fibula	Vertebra	**Ductus Deferens**	Sclera
Humerus	Vertebral	Mucosa	Unilateral
Intervertebral disc	**Brain**	Muscularis	**Gallbladder**
Joint	Bilateral	Unilateral	Lumen
Left	Brain stem		Mucosa
Ligament	Cerebellum		Serosa
Lumbar	Cerebrum		Wall
Mandible	Choroid plexus		
Maxilla	Cranial nerve		
	Left		
	Meninges		

(*Continued*)

Table 19.3 (*Continued*)   Topographical Terminology (Organ and Site) Used to Locate Gross Lesions

Harderian Gland
Bilateral
Left
Right
Unilateral

Heart
Aortic valve
Atrium
Left atrium
Right atrium
Atrioventricular valve
Endocardium
Epicardium
Mitral valve
Myocardium
Pericardium
Pulmonic valve
Septum
Tricuspid valve
Valve
Ventricle left
Ventricle right
Ventricle

Large Intestine
Anus
Ascending colon
Cecum
Descending colon
Lumen
Lymphoid tissue
Mucosa
Rectum
Serosa
Transverse colon
Wall

Small Intestine
Duodenum
Ileum

Jejunum
Lumen
Lymphoid tissue
Mucosa
Serosa
Wall

Kidney
Bilateral
Capsule
Cortex
Left
Medulla
Papilla
Pelvis
Right
Unilateral

Lacrimal Gland
Bilateral
Extraorbital
Intraorbital
Left
Right
Unilateral

Larynx

Liver
Anterior
Bile duct
Capsule
Caudate lobe
Left lateral lobe
Median lobe
Parenchyma
Posterior
Right lateral lobe

Lung
Apical lobe
Bilateral

Bronchus
Cardiac lobe
Diaphragmatic lobe
Intermediate lobe
Left
Parenchyma
Pleura
Right
Unilateral

Lymph Node
Axillary
Bilateral
Bronchial
Deep cervical
Hemal
Iliac
Inguinal
Left
Lumbar
Mandibular
Mediastinal
Mesenteric
Pancreatic
Popliteal
Prefemoral
Renal
Right
Thoracic

Mammary Gland
Abdominal
Bilateral
Cervical
Inguinal
Left
Right
Thoracic
Unilateral

Mesentery
Fat

Nose
Bilateral
Left
Nares
Right
Septum

Ovary
Bilateral
Capsule
Left
Parenchyma
Right
Unilateral

Oviduct
Bilateral
Left
Right
Unilateral

Palate

Pancreas
Parenchyma
Serosa

Parathyroid Gland
Bilateral
Left
Right
Unilateral

Penis

Peripheral Nerve
Brachial plexus
Plantar
Radial

(*Continued*)

Table 19.3 (*Continued*)   Topographical Terminology (Organ and Site) Used to Locate Gross Lesions

Sciatic	**Skeletal Muscle**	**Scapula**	**Unilateral**
Spinal	Abdominal	Scrotal	
Tibial	Anterior	Scrotum	**Thymus**
Trigeminal	Back	Site of application	Capsule
Ulnar	Bilateral	Mass	Parenchyma
	Diaphragm	Subcutaneous tissue	Unilateral
Pharynx	Dorsal	Tail	
	Forelimb	Thoracic	**Thyroid Gland**
Pituitary Gland	Head	Unilateral	Bilateral
	Hind limb	Ventral	Capsule
Preputial Gland	Lateral	Vulva	Left
Bilateral	Left		Parenchyma
Left	Mandibular	**Spinal Cord**	Right
Right	Neck	Canal	Unilateral
Unilateral	Posterior	Cervical	
	Right	Dura	**Tissue NOS***
Prostate	Thoracic	Lumbar	Abdominal
Bilateral	Unilateral	Parenchyma	Anterior
Dorsal	Ventral	Thoracic	Cranial
Left			Dorsal
Parenchyma	**Skin**	**Spleen**	Left
Right	Abdominal	Capsule	Mediastinum
Serosa	Anterior	Parenchyma	Nasal
Unilateral	Back	Red pulp	Oral
Ventral	Bilateral	White pulp	Pelvic
	Control		Pericardial
Salivary Glands	Dorsal	**Stomach**	Posterior
Bilateral	Face	Forestomach	Right
Left	Foot	Glandular	Scrotal
Parotid gland	Forelimb	Lumen	Thoracic
Right	Hair	Mucosa	Ventral
Sublingual gland	Head	Serosa	
Submandibular gland	Hind limb	Wall	**Tongue**
Unilateral	Inguinal		Anterior
	Lateral	**Testes**	Dorsal
Seminal Vesicle	Left	Bilateral	Mucosa
Bilateral	Lip	Capsule	Parenchyma
Left	Lumbar	Left	Posterior
Parenchyma	Neck	Parenchyma	Ventral
Right	Nipple	Right	
Serosa	Posterior		
Unilateral	Prepuce		
	Right		
	Sacral		

(*Continued*)

Table 19.3 (*Continued*)   Topographical Terminology (Organ and Site) Used to Locate Gross Lesions

Tooth	Mucosa
Bilateral	
Gingiva	**Right**
Incisor	Serosa
Left	Unilateral
Lower	Wall
Molar	
Right	**Vagina**
Upper	Lumen
	Mucosa
Trachea	Wall
Lumen	
Mucosa	**Zymbal's Gland**
Wall	Bilateral
	Left
Ureter	Right
Bilateral	Unilateral
Left	
Right	
Unilateral	
Urethra	
Bulbourethral gland	
Distal	
Proximal	
Urinary Bladder	
Lumen	
Mucosa	
Serosa	
Wall	
Uterus	
Bilateral	
Body	
Cervix	
Horn	
Left	
Lumen	

Notes: Use the organ name from the gross topography hierarchy level table or use tissue "not otherwise specified" (NOS). Use the most appropriate site for the organ from the table. If clarification is needed, "free text" can be entered into the "notes" section on the individual animal necropsy record.

Table 19.4   Veterinary Anatomical Terms

Anterior—Undesirable use in veterinary anatomy

Caudal—Pertaining to the tail

Coronal Plane—A plane at right angles to a sagittal plane. Dividing the body into dorsal and ventral portions. Sometimes referred to as the frontal plane

Cranial—Pertaining to the head

Distal—Situated away from the center of the body or from the point of origin

Dorsal—Pertaining to the back or upper surface. Nearer the back surface of the body

Lateral—On the side. Farther from the median or midsagittal plane

Medial—Relating to the middle or center

Posterior—Undesirable use in veterinary anatomy

Proximal—Situated toward the center of the body or toward the point of origin

Rostral—Pertaining to the nose. In a direction toward the nose (rostrad)

Sagittal plane—A plane at right angles to a coronal plane. The midsagittal (or median) plane divides the body into left and right halves. In contemporary usage, used for any plane parallel to the median, i.e., as a synonym for parasagittal

Transverse—Crosswise; lying across the long axis of the body or of a part

Ventral—Pertaining to the undersurface. Situated nearer the undersurface of the body

TISSUES COLLECTED DURING NECROPSY

There is a "best practices" list that has been published by the Society of Toxicologic Pathology enumerating the tissues that should be collected from GLP-quality repeat dose toxicity studies that are supportive of the registration of new pharmaceutical products (Bregman et al., 2003; U. S. Food and Drug Administration [FDA], 1988). These tissues would include but are not limited to adrenal gland, aorta, bone (nonrodents, sternum or rib; rodents, femur with articular cartilage) with bone marrow, brain, cecum, colon, duodenum, epididymis, esophagus, eye with optic nerve, gall bladder, Harderian gland, heart, ileum, jejunum, kidney, liver, lung (with main stem bronchus), lymph nodes (mandibular, mesenteric), mammary gland (female), ovary, pancreas, parathyroid gland, peripheral nerve (sciatic), pituitary, prostate, salivary gland, seminal vesicle, skeletal muscle (thigh), skin, spinal cord (cervical, midthoracic, lumbar), spleen, stomach, testes, thymus, thyroid gland, trachea, urinary bladder, uterus, vagina, and any tissues or organs whether listed or not that exhibit gross lesions and any tissue masses. This tissue list can be used for all types of repeat dose toxicity studies, regardless of duration, route of administration, species or strain of animal, or the type of test article. However, the route of administration may suggest that additional tissues be collected, such as nasal cavity, nasal turbinates, pharynx, larynx, and tracheobronchial lymph nodes. Tissues that are known targets of the test article and are not included on this basic list should be added to the list. Finally, the presence or absence of some organs or tissues might be unique to a given species and they should be added to or deleted from the list as is necessary. Some specific examples are minipigs (spiral, transverse, and descending colon, vesicular gland instead of seminal vesicles, bulbourethral gland [urogenital], and no parathyroids), rats (no gallbladder), rabbits (appendix, sacculus rotundus), dog (no seminal vesicles), and nonhuman primates (the diaphragm, axillary lymph nodes, tracheobronchial lymph nodes, tonsils, lip, and the stomach, which can sometimes be listed only by its component parts [cardia, fundus, and pylorus]).

Please note that every tissue or organ is not represented in the aforementioned list, because agencies expect only a representative sampling to maximize detection, resources, and safety. There are additional tissues that are recommended for collection and evaluation by other regulatory agencies and these include optic nerve, oviduct, ureter, nasal cavity, Zymbal's gland, clitoral, preputial gland, diaphragm, extraorbital lacrimal gland, rectum, three different salivary glands, larynx,

pharynx, coagulating gland, and tongue. The reasoning for the lack of inclusion of these tissues into the basic core list has multiple considerations. First, historical data support the fact that expressions of toxicity or neoplasia rarely if ever occur in these additional tissues. Second, tissues currently on the list already provide adequate screening for specific organ systems (e.g., single salivary gland vs. three salivary glands). Third, while routine examination may not be required for some tissues, gross examination may suggest the addition of other tissues to the list. Fourth, some animal tissues such as Zymbal's gland, clitoral/preputial glands, etc., do not have human counterparts, and therefore findings in these tissues or organs do not translate to human safety.

NECROPSY PROCEDURES BY SPECIES

Gross

Prior to the initiation of any dissection or necropsy if there are any questions with regard to anatomy, appropriate text books should be consulted (Ankel-Simons, 2000; Berringer et al., 1968; Evans, 1993; Getty 1975a,b; Gilbert, 1968; Gray, 1985; Nickel, 1986; Popesko et al., 1992a,b; Schummer, 1977; Schummer et al., 1979, 1981). Even if there is not a sufficiently complete anatomical reference for a particular species, suitable extrapolations can generally be made from detailed reference material dealing with other species.

Generally speaking, the necropsy initiates with the euthanasia of animals. While a variety of different techniques exist, it should be completely understood that animals need to be euthanized in a humane and compassionate manner (AVMA, 2007; Hellebrekers and Hedenqvist, 2011; NRC, 2011). Procedures vary by species and situation but there is one unifying concept and that is animals be shown dignity and respect in their final moments and with their remains. It is well recognized that animals endure stress when other animals are euthanized in the presence of their comrades. Accordingly, every effort should be made to euthanize animals away from the rest of the group or groups. Furthermore, the technician performing the euthanasia should not be covered with blood and biological fluids or tissue debris. Aside from the ethics of such a protocol, the elimination of this stress from animals will have a positive effect on a variety of physiological parameters, thereby improving the quality of the clinical pathology data coming from the study (Doerning and Cruze, 1997).

The following is the procedure for gross observations only in necropsy of rats, mice, guinea pigs, hamsters, rabbits, dogs, cats, ferrets, swine, and nonhuman primates. These procedures may vary slightly in sequence or may be modified based upon a specific protocol or sponsor requirements. Tissues to be weighed are determined by the study protocol. Tissues are saved in fixative as specified in the study protocol (typically 10% NBF). If data are collected online, organs or tissues that are not specified through the program being used are considered recorded under the heading of other. Confirm the animal's identification and save such if stated in the protocol. If identification is missing or unreadable, this must be confirmed by other study personnel, and a cage card or other methods of identification need to be retained with tissues. Examine, palpate, and record all external abnormalities. Make a ventral midline incision in the skin and underlying muscle from the pelvic symphysis to the sternum. Sever the ribs at the costosternal junction on one side and continue a skin incision to the point of the mandible. Reflect the skin and rib cage as needed to observe underlying tissues and organs. Inspect the submandibular lymph nodes, salivary glands, and thyroid/parathyroid glands. Look at the thoracic cavity and its contents. Examine the abdominal cavity and its contents, reflecting the intestinal tract for inspection as needed of the proximally and dorsally located organs (kidneys, adrenals). Inspect the pelvic organs and gonads. In rodent, males express the testes from the scrotum into the abdominal cavity. In the event of finding abnormalities, a more

detailed dissection of the organ should be performed. If the presence of any gross lesion is noted, a determination by the pathologist, necropsy supervisor, or study director will be made as to whether any lesion, tissue, or organ is to be saved for later further evaluation.

Some studies may require a gross examination of the nervous system only, especially if clinical observations warrant the following: (1) Expose the skull by reflection or removal of the skin and muscle. Remove skull cap and expose the brain. Sever the brain at the olfactory bulbs and reflect the brain superiorly. Inspect the brain and pituitary. (2) Expose the right lateral thigh muscle by incising the skin and subcutis of the right leg. Separate the thigh muscles and expose and inspect the sciatic nerve. The examination may be extended by incising the skin and subcutis in the axillary areas and also inspecting the brachial plexus and/or by removing the cervical, thoracic, and lumbar sections of the spinal column, splitting the vertebral arches, and exposing the spinal cord for inspection and potential fixation.

Full or Detailed

The following is a proposed procedure for the complete necropsy and collection of the complete gross observations for rodents (e.g., rat, mouse, and hamster). These procedures may vary slightly in sequence or may be modified based upon a specific protocol or sponsor requirements. Tissues to be weighed are determined by the study protocol. Tissues are saved in fixative as specified in the study protocol (typically 10% NBF). If data are collected online, organs or tissues that are not specified through the program being used are considered recorded under the heading of other. The first thing that one should do is to confirm the animal identification and save such. If the individual animal identification is missing or illegible, it must be confirmed and documented by other study personnel. A cage card or other methods of identification are always retained with tissues. Write the animal's identification number on each labeled cassette for small or paired organs. Next, examine, palpate, and record any and all external abnormalities. A ventral midline incision should be made through the skin and subcutis from the pelvic symphysis to the point of the lower jaw (mandibular symphysis). Reflect the skin to expose underlying muscle and neck organs. Remove any injection sites or treated sites and a nontreated control site (if required by protocol). For cases involving the skin or subcutis, place treated and corresponding control sites on blotter paper or cardboard. Remove the salivary glands and associated lymph nodes together. If salivary glands are required by protocol to be weighed, dissect these away from the attached lymph nodes. Remove a section of the skin to include mammary glands from the inguinal region. Make a ventral midline incision in the abdominal musculature from the pelvic symphysis to the sternum. Remove the sternum at the costal junction, trim, and save. Split the symphysis of the mandible and remove in toto the tongue, trachea, larynx with thyroid/parathyroid, esophagus, thymus, heart, aorta, and lung. Palpate and inflate or infuse the lungs with fixative. If to be weighed, remove the heart and express the contained blood. Expose the thyroid and parathyroid glands. Remove the liver. If the liver is to be weighed, be sure to excise the gallbladder in mice. For rats, make multiple deep incisions into the liver and remove one or more lobes to be saved. For mice, incise and save all lobes. Remove the spleen. Remove the gastrointestinal tract from the stomach (gastroesophageal junction) through the terminus of the distal colon, including the mesentery and mesenteric lymph nodes, in toto. Trim away mesentery and mesenteric fat. The stomach should be incised and opened along the greater curvature and the mucosal surfaces examined. Make incisions into the cecum and gently express the contents. Inject 10% NBF into the various sections of the gastrointestinal tract (5 or 10 mL syringe with 18–22 G needle). Remove the adrenals, trim away any extraneous tissue, and place into a cassette. Both kidneys should be removed and again any extraneous tissue trimmed away. For the left kidney, an incomplete incision is made into the kidney longitudinally from pole to pole, and on the right kidney, an incomplete transverse incision is made into the kidney near the hilus. Both testes are removed, extraneous tissue trimmed, and the epididymis removed from each testis.

If necessary for weighing, trim the epididymis away from any extraneous tissue. If the epididymides are not to be weighed, they can be left intact with the seminal vesicles, prostate, urinary bladder, etc. Split the pubis and remove in toto the seminal vesicles, coagulation gland, prostate, urinary bladder (should be inflated with 10% NBF), and rectum. Remove and trim the ovaries if needed for weighing. Otherwise remove in toto the ovaries, uterus, cervix, vagina, and urinary bladder (should be inflated with 10% NBF) and rectum. For rats, remove skeletal (thigh) muscle from the posterior aspect of the right leg. For mice, leave the muscle attached to the femur. In rats, remove the sciatic nerve from the right leg and place in a cassette or leave attached to the skeletal (thigh) muscle. When dealing with mice, leave the sciatic nerve attached to the leg skeletal muscle. The right femur should be transected near the head. For rats, the skin and muscle of the right leg are removed, and the tibia is transected at a point near the tibiotarsal joint (knee with articular surfaces). For mice, the skin is merely removed. The leg muscles and sciatic nerve remain attached, and the tibia is transected near the tibiotarsal joint (knee with articular surfaces is saved for evaluation). Bone marrow smears, if required, are prepared from the left leg. Expose the cranial and nasal bones by incising the caput skin and reflecting it laterally. Examine the exorbital lacrimal glands and ear canals. Remove the eyes, Harderian glands (if possible), and Zymbal's gland. Remove the skull top and expose the brain. Sever the spinal cord at the "foramen magnum" and carefully remove the brain intact. The pituitary should be carefully removed from the floor of the "calvaria." If the eyes as per the protocol are to require a special fixation, remove the eyes and Harderian glands from the orbit and place in a cassette. The cervical, thoracic, and lumbar vertebrae should be removed with the spinal cord intact, trimming away any excess muscle. Incompletely transect the vertebral column in two places (one-third intervals) to facilitate fixation of the spinal cord. For mice only, after harvesting all required tissues, wrap "the pelt" with carcass remains in gauze or cutting paper and place this all in a labeled specimen jar. For rats, after harvesting all required tissues, wrap "the pelt" with carcass remains in cutting paper, "write" the animal number on outside, and place in a waste bag provided. Discard all remains properly in a suitably colored and labeled waste bag (i.e., red bag labeled "biohazard").

The following is a proposed procedure for the complete necropsy and collection of the complete gross observations for rabbits, guinea pigs, cats, dogs, ferrets, swine, and nonhuman primates. These procedures may vary slightly in sequence or may be modified based upon a specific protocol or sponsor requirements. Tissues to be weighed are determined by the study protocol. Tissues are saved in 10% NBF or other fixative as specified in the study protocol. If data are collected online, organs or tissues that are not specified through the program being used are considered recorded under the category of other. Before doing anything, confirm the animal's identification and save the ear tag or tattoo. If these are missing or unreadable, this must be confirmed and documented by other study personnel. The cage card or other methods of identification are also retained with the tissues. Write the animal's identification number on each labeled cassette for small or paired organs. Examine, palpate, and record all external abnormalities. Remove a longitudinal skin section or a thin strip of skin along the ventral midline extending from the cervical region to the genital area. For dogs and swine, remove the mandibular salivary glands and a pair of cervical lymph nodes. For rabbits, remove the submaxillary salivary glands and a pair of cervical lymph nodes (if these organs are required by the protocol). Remove a section of skin to include mammary gland from the inguinal area (include the nipple of the gland). Open the abdominal cavity with a longitudinal incision extending from the base of the xiphoid cartilage to the pubis. Open the thoracic cavity by cutting through the costochondral junctions on both sides of the sternum and remove the rib cage. Extraneous tissue is trimmed from the sternum and at least four sternebrae are removed from the middle of the sternum, if this is to be saved. Examine all thoracic and abdominal organs in situ before proceeding. For dogs and swine, remove the thyroids and parathyroids, trim away any extraneous tissue, and place in a cassette. For rabbit and nonhuman primate, leave these glands attached to the trachea. The tongue, larynx, trachea, esophagus, lung, heart, aorta, and thymus are

removed in toto. Palpate the lungs and inflate or infuse with fixative. Open the pericardial sac and express any blood that is present. Dissect the heart free from the thoracic "pluck," if it is required to be weighed. For nonhuman primates, dissect away the trachea and bronchial lymph nodes from the thoracic "pluck" if they are to be weighed and placed in a cassette. Remove a section of ribs with the costochondral junctions from the rib cage (if stipulated by the protocol). The liver should next be removed. Incise the gallbladder and drain the bile. Make multiple incomplete but deep incisions into the various lobes of the liver, or the pathologist or necropsy supervisor will collect any required sections. The adrenals should next be removed, trimmed of any extraneous tissue, and placed in a cassette. The kidneys are next removed and trimmed of any extraneous tissue. Identify the left kidney by making an incomplete longitudinal incision from pole to pole and identify the right kidney by making an incomplete transverse incision between the poles in the general area of the hilus. Next, the entire gastrointestinal tract is removed to include the stomach, small intestine, colon and attached spleen, pancreas, and mesenteric lymph nodes. Remove the spleen and make two incomplete incisions equidistant from one another (at one-third intervals), or for dogs, remove a strip that is one centimeter in width and the width of the spleen in length from the larger area of the spleen. The stomach along with the duodenum and pancreas are removed. Incise the stomach along the greater curvature and rinse away the contents with normal saline. Examine the mucosa of the stomach and duodenum. For dogs and swine, strip the jejunum from the mesentery, carefully open it, and examine the mucosal surface. Remove and save a 10–20 cm piece. For dogs and swine, remove the ileum, cecum, colon, and mesenteric lymph nodes. Open these tubular organs and rinse away the contents with normal saline and examine the mucosal surfaces. With rabbits, remove the mesenteric lymph nodes with a segment of the descending colon (forms a "horseshoe" circle around the lymph nodes). Remove an 8 cm in length piece of appendix with jejunum attached. As a single unit, remove a length of 10–20 cm of terminal ileum (with Peyer's patch), 10–20 cm length of the initiation of the ascending colon, and a small segment of the cecum with the "sacculus rotundus" attached. Open the cecum, rinse away the contents of the mucosa with normal saline, and examine. For males, remove the testes and epididymides. Trim the epididymides from the respective testes. Split the pubis and remove the prostate, urinary bladder (with a longitudinal incision for fixation), and rectum. Note that for rabbits, the pelvic cavity "must" be opened completely to examine the male accessory reproductive organs and to obtain sections of the prostate and the seminal vesicles. For females, remove the ovaries, trim away any extraneous tissue, and place in a cassette. Split the pubis and remove the uterus, cervix, vagina, urinary bladder (with a longitudinal incision for fixation), and rectum. Expose the lateral thigh muscles by incising the skin and subcutis of the right leg. Remove a section of muscle with the sciatic nerve. Remove the femur including the articular surface of the knee joint. If a bone marrow smear is required, the left femur is cleaned of muscle and extraneous tissue and transected immediately distal to the head of the femur with a Stryker saw. For dogs, nonhuman primates, and swine, remove the lacrimal glands or the third eyelid, if present, and both eyes with approximately 0.5 cm of optic nerve attached. For rabbits, remove the Harderian gland/lacrimal gland and both eyes. The nasal cavity is not routinely opened for examination. Remove the head from the carcass and expose the skull by removal of the skin and muscle from the cranium. Remove the "calvaria" and expose brain. Carefully remove the brain and the pituitary. Place the pituitary in a cassette. If required by the protocol, remove a portion of the nasal turbinates. (Note that the pituitary gland in the rabbit is covered by a thin plate of bone that must be carefully dissected away in order to expose the gland. Cut the bone at each side and then on the posterior border and carefully lift the bone away from the gland. Place the pituitary in a cassette.) Remove the cervical, thoracic, and lumbar sections of the vertebral column containing the spinal cord (2–3 vertebrae from each area). After harvesting all required tissues, place the carcass with all tissue remains and the animal's cage card in suitably colored and labeled waste bag (e.g., red plastic bag labeled "biohazard"). Discard the remains.

ORGAN WEIGHTS

An extremely important part of the necropsy and pathology evaluation is the collection of organ weights (Sellers et al., 2007). Organ weights are a commonly used parameter in toxicology evaluations. Ideally, a complete understanding of a test article's metabolism, pharmacokinetics, and mechanism of action coupled with an understanding of the biochemistry, molecular biology, and physiology of the animal model will aid in predicting and understanding potential organ weight changes. If organs are not to be weighed, they should be placed in fixative immediately. But if organs are to be weighed, they should be kept moist by misting with normal saline until they are weighed. Organs that exist in pairs (e.g., adrenals, kidneys, etc.) should be weighed together and not individually. Keep in mind to never compare the weights of fixed versus fresh organ weights of the same tissues.

Scales

Scales in a necropsy room should always be checked and calibrated before any weighing activity. Allow the balance to warm up for at least 15 min before checking. Press "tare" to zero the balance. Place relevant test weights encompassing the weighing range on the balance pan. Adjust the sensitivity adjustment until the weight indicator corresponds exactly to the magnitude of the applied calibration weights for both the low end of the weighing range and the high end of the weighing range. Verification of a balance's accuracy should be documented not only by the person weighing the organ but also by another individual involved in the necropsy, initialed and dated in the scale calibration record book kept in the necropsy room. When tissues are required by the protocol to be weighed prior to fixation at necropsy, the following procedure should be used: (1) Remove the organ to be weighed from the animal. (2) Trim away any excess fat and connective tissue from the organ or tissue. (3) Place the material to be weighed in a weigh boat that has been identified with the animal's number or in separate compartment in the individual animal tissue collection tray, keeping the tissue moist (not immersed) with saline. (4) Place the organ on a tared balance. (5) Record the weight on a paper or electronic necropsy sheet or other authorized form. (6) Return the tissue or organ to the collection tray. If tissues are required by the protocol to be weighed "postfixation," but prior to gross trimming, the following procedure should be used: (1) Remove the tissue or organ to be weighed from the tissue container. (2) Trim away any excess fat and connective tissue from the tissue or organ. (3) Place material on a piece of weighing paper, which has been identified with the animal's number. (4) Place tissue or organ on a tared scale. (5) Record the tissue or organ weight on a paper or electronic necropsy sheet or other authorized form. (6) Return the organ to the formalin-filled container. If the protocol stipulates that postfixation is to be performed at a contract histology laboratory, then that laboratory's SOP should be followed.

Procedures

The selection of the balance to be used at the time of weighing is tissue and species dependent. Tissues should be weighed and recorded according to the different sensitivities (Table 19.5). Sensitivities for other species/organs may well be specified by the pathologist, pathology associate, or study director. Again, paired organs should be weighed together unless specified by the pathologist to be weighed separately. For species with gallbladders, remember that the liver weight includes incised gallbladder and this needs to be documented.

Toxicology studies of GLP quality should contain organ weight data for the liver, heart, kidneys, brain, adrenal glands, and testes (best if from sexually mature animals) from animals in repeat dose studies from 7 days to 1 year's duration. Only calibrated balances should be used,

Table 19.5   Balance Sensitivities by Organ and Species

	Rats/Mice (g)	Dogs/Rabbits/Swine/Nonhuman Primates (g)
Spleen	0.01	0.1
Kidney	0.01	0.1
Liver	0.01	0.1
Brain	0.01	0.1
Heart	0.01	0.1
Prostate	0.01	0.1
Lung	0.01	0.1
Adrenal(s)	0.001	0.01
Thyroid/parathyroid(s)	0.001	0.01
Ovary(ies)	0.001	0.01
Testis(es)	0.001	0.01
Epididymis(ides)	0.001	0.01
Seminal vesicles	0.001	0.01
Thymus	0.001	0.01
Pituitary	0.001	0.01
Salivary gland(s)	0.01	0.1
Uterus	0.01	0.1

with a weight range that comfortably encompasses the weights of the organs that will be weighed on it. Ideally, the average organ weight should be within the mid-third segment of the calibration curve for the balance. Some organs such as the liver may actually demonstrate changes in weight in studies of less than 7 days' duration, so the weighing of organs may actually be a very sensitive indicator of toxicologic activity. Changes in brain weights are not usually associated with the development of neurotoxicity; however, the value of brain weights is in the calculation of organ-to-brain-weight ratios. So if terminal body weights of animals are highly variable as a result of test article effects, the use of organ-to-brain-weight ratios can significantly reduce numeric scatter. Indeed, organ-to-brain-weight ratios should be routinely determined for all repeat dose toxicity studies. Changes in heart weights may well be predictive of the development of myocardial hypertrophy that could be extremely difficult if not impossible to pick up with macroscopic or even microscopic examination. Similarly for the liver, increases in weight may be reflective of hepatocellular hypertrophy secondary to peroxisome proliferation or enzyme induction. Changes in kidney weight may indicate renal toxicity or tubular hypertrophy. Finally, changes in the weight of the adrenal glands may indicate test article toxicity, stress, hyperplasia, hypertrophy, or a general endocrinopathy.

Thyroid and pituitary gland weights should be collected for all species except mice. When dealing with rodents, the fixation of thyroid and pituitary glands before weighing may lead to the generation of more accurate weights. Weighing the thyroid and pituitary glands in mice, because of their sizes, may lead to artifacts that confound microscopic data interpretation.

Inflammation or changes in the production of sperm may be reflected in weight changes for the epididymides. Testicular weight changes may indicate the presence of edema or changes in the seminiferous tubules. Prostate weights can be affected by test articles demonstrating androgenic, antiandrogenic, or estrogenic activity. In repeat dose toxicity studies involving rats, the testes, epididymides, and prostate should always be weighed. Testes should always be weighed in mice, but the prostate and epididymides are weighed on an individual basis only. For male rodents, the weights of seminal vesicles will add little if any value to impressions gained and conclusions drawn from prostate is weighed. For nonrodents, testes should always be weighed, but the epididymides and prostate should only be weighed on an individual basis. It is important to recognize that the

weights of the prostate, epididymides, and other male accessory organs are only of significant value when the organs are collected from sexually mature animals.

For GLP repeat dose toxicity studies in rodents of durations greater than 7 days, splenic and thymic weights should always be captured. For nonrodents, post pubertal dogs, and nonhuman primates, thymic weights can provide valuable information, but the normal involution of the thymus can complicate the interpretation of thymic weight data, especially when studies are greater than 3 months' duration. Splenic weights in nonrodents can be influenced by the quality of exsanguinations and the method of euthanasia. Lymphoid organ weights can exhibit a high degree of variability for a variety of reasons, and so the weights of spleens and thymi need to be viewed concurrently with their histomorphologic evaluation. General lymph nodes are difficult to dissect from all fats and can also vary markedly in size, making the collection of weights from these structures a task of questionable value.

There are some organs that are not routinely weighed but can be weighed depending upon the activity of the test article. Histopathology is typically sufficient to detect toxicity in salivary glands, but if a test article has specific effects on secretory glands, then weighing them might be an appropriate action. The pancreas is isolated only with extreme difficulty in rodents but is readily isolated from nonrodents, so weighing may not be appropriate in the former case but could be useful in the latter case. The gastrointestinal tract can be very problematic. Dissection and trimming of the gastrointestinal tract tend to be very inconsistent and when coupled with the inconsistent filling of the gut (if not emptied and cleaned), make weight collection data highly variable. The uterus and ovaries can be weighed if necessary, but are not organs that are routinely scheduled for weighing. Lung weights can provide important information in inhalation studies. However, if the route of administration is by some pathway other than inhalation, then the collection of lung weights is not necessary, and adequate evaluation is gained from the histomorphological analysis. Organ weights should not be collected from animals that are euthanized or suffer untimely deaths. This is because there are no matched controls and there might be significant differences in nutritional state, congestion, etc. that will undoubtedly confound the data interpretation. Finally, the study pathologist should have the opportunity to review all of the organ weight data and provide his or her input to the interpretation of such data. The study pathologist is really the most qualified person to evaluate organ weight changes and correlate them with the clinical chemistry, hematology, gross pathology, and histomorphology.

SPECIAL NECROPSY PROCEDURES

Eye Removal

Not uncommonly, a more detailed dissection of the eye will be requested (Maggs et al., 2008; Slatter, 2001). Regardless of species, the removal of the eyes commences with the removal of the conjunctiva. The palpebral conjunctiva is the pink tissue that lines the inner surface of the eyelids. The conjunctiva is elevated with a pair of forceps and trimmed free of or off the inner surfaces of the upper and lower eyelids. The bulbar conjunctiva is the loose tissue that attached to and surrounds the sclera (white part of the globe). This tissue is elevated with forceps and dissected free or off of the globe. The eye is then carefully removed with a pair of forceps, transecting the optic nerve at a point as distally as possible from the eye. After the eye with its optic nerve attached is removed, remove the aqueous humor. This is accomplished by inserting a 1 mL syringe with 25–27G 1 in. needle into the anterior chamber of the eye. As the needle is being inserted into the anterior chamber, simultaneously pull back on the syringe plunger so that the aqueous humor will steadily flow into the syringe. Next, remove the cornea. This is done by making a deep incision that follows the demarcation between the sclera (white part of the eye) and the cornea (clear part

of the eye). This incision should follow the complete circumference of the eye's corneoscleral junction. Trim with a small pair of scissors as is necessary and gently lift and remove the cornea. Next, trim the iris and ciliary body from the eye and free and remove the lens. Remove and collect the vitreous humor (gel-like substance behind the lens) using a 1 mL syringe and a 20–22G needle or a small spatula. Peel or scrape the choroidal–retinal tissue from the posterior interior surface of the globe. (Note that in the rat, the retina often comes out with the vitreous humor.) One can tell the difference between the two tissues by the fact that the vitreous humor is clear and colorless and the retinal tissue is pink. Trim the optic nerve from the rear of the exterior of the eyeball. If the pigmented portion of the retina is also required, the posterior portion of the globe will be submitted intact with or without the neuroretina, since the pigmented layer of the retina is generally tightly adhered to the sclera.

Blood Smears

If a protocol requires blood or bone marrow smears to be made and does not specify from which location, the following procedure can be used. To prepare a blood smear from a rodent, anesthetize or asphyxiate the animal by carbon dioxide asphyxiation and place the animal in dorsal recumbency. Collect blood using a syringe and needle (3 mL syringe and 22G 1 in. needle) via percutaneous cardiocentesis. Alternatively, open the chest, expose the heart, and collect blood via direct cardiocentesis. For other species, blood can be collected via access to any suitable peripheral vein. Place a single drop of the collected blood onto a glass slide near the end of the slide, about 1 cm from the edge (slide 1). Take a second glass slide (slide 2) and hold it at an approximately 45 angle to the slide with the blood on it (slide 1) and set the edge into the blood drop. The drop of blood will spread out into a line underneath the edge of slide 2 as a result of capillary action. Now quickly push slide 2 over slide 1 in one continuous steady movement to the opposite end of slide 1, being sure to maintain the initial angle between the two slides. In this way, a uniform smear with a progressively developing feathered (thinning) edge will be produced. For more details on blood smear preparation, references are readily available (Bessis, 1977).

Bone Marrow Smears

Bone marrow smears can be prepared for all species according to the following procedure. Moisten a 2/0 natural sable paintbrush with normal phosphate-buffered saline (PBS) and blot off any excess liquid. Remove a femur (all species) or rib (large species) from the animal. Expose the marrow cavity by breaking or cracking the femur at a point that is midshaft or a rib at any point between the thoracic vertebrae and costochondral junction for that rib. Introduce the premoistened paintbrush into the red marrow and gently and slowly twirl it. Take the impregnated brush and move it down the length of a glass slide in a slightly wavy linear pattern using a continuous motion. Continue by making a second wavy line next to the first on the same slide. Repeat this on the second slide. (Often it is not necessary to dip the brush back into the marrow for the second slide. There are usually a sufficient number of bone marrow cells that remain on the brush.) Allow the slides to air-dry away from formalin fumes. Use a clean brush for each animal. To clean brushes, agitate the brushes in two changes of PBS and then rinse again with fresh PBS before use. When brushes become worn out (leaving hairs or bristles on the slide), replace with new brushes.

Sterile Procedure

It would be an incredibly rare event for a necropsy to be performed under sterile conditions. This type of procedure would most likely be invoked only in cases where there is a severe complicating fungal infestation, bacterial infection or viral infection, and a highly infectious or toxic test article

(e.g., live virus) or in cases where samples are to be collected for PCR analysis. There are many resources available in addressing aseptic technique, and for details, the reader should consult one (Slatter, 1993).

PCR Analysis

Occasionally, especially if working with gene therapy compounds, where PCR analysis will be required as part of the study protocol, a necropsy will need to be performed in such a fashion as to preclude the intertissue contamination with foreign nucleic acids from other tissues (Compton and Riles, 2001; Feinstein and Waggie, 2011). While many aspects of the necropsy will remain unchanged from a standard necropsy, molecular techniques such as PCR analysis are of very high sensitivity, and steps must be taken to eliminate the potential for cross-contamination of nucleic acids between specimens collected for such analysis. Strict control of the environment is necessary to prevent potential contamination of the necropsy table and bench surfaces. One way to address this is for necropsy technicians to collect their samples from animals, tissues, or organs for PCR analysis in laminar flow hoods. As a general rule for the collection of samples for PCR analysis, no instrument should touch any more than a single point of a single tissue or organ at any time. Gloves need to be changed frequently, at a minimum between tissues or organs. Table coverings or paper need to be changed from one tissue or organ to another. Instruments need to be continually replaced and replenished with fresh, clean instruments or instruments that are sterilized by heat or submerged in a 10% solution of bleach or some other suitable disinfectant for a period of contact time of 5 min and rinsed (PBS) and dried before using again in sampling another tissue or organ. Any specimens collected should be immediately placed in sterile tubes or containers and frozen rapidly at $-80°C$.

Perfusion Fixation

Another specialty technique that may be used occasionally in necropsy is whole animal or isolated organ vascular perfusion fixation (McMenamine, 2000; Rostgaard et al., 1993; Scouten et al., 2006; Simmons et al., 1996; Stickrod and Stansifer, 1981; Stretch et al., 1999; Spector and Goldman, 2005). While when this is done it is usually performed in rats or mice, it can be performed in pretty much any species. The advantages of this procedure are the following: (1) Fixation begins immediately after the arrest of systemic circulation, minimizing the alteration of cell structure resulting from "postmortem" effects. (2) Vascular perfusion under in situ conditions results in a uniform and rapid dissemination of fixative into all parts of the tissue via the vascular bed, resulting in an increased depth and rate of actual fixation. (3) The manipulation of tissues after circulatory arrest and prior to fixation is minimized thereby resulting in fewer artifacts. (4) Many organs and tissues can be fixed concurrently. (5) For immunocytochemical procedures utilizing relatively mild fixation conditions, fewer changes from autolysis result; greater immunocytochemical activity is retained, and the redistribution or translocation of cellular components is minimized. There are a variety of possible routes for vascular perfusion and they would include (1) the liver (portal vein), (2) central nervous system including pituitary (aorta via the left ventricle), (3) the kidney (descending aorta), and (4) the whole animal (descending aorta or vena cava).

Virtually any organ-specific organ perfusion procedure can be readily modified to effect fixation (Mehendale, 2008). Typically, the perfusion procedure is broken into two parts, the preperfusion portion and the actual perfusion portion. For preperfusion, the animal is weighed and injected with a suitable anesthetic. An inhalational anesthetic such as Metofane can be used also. Before beginning the perfusion procedure, animals should be checked for a suitable plane of anesthesia by a person that is qualified to perform such an assessment. There should be no deep pain reflex present in the animal. During the perfusion procedure, every attempt should be made to keep the animal

warm (body temperature), as in this anesthetized state the animal will be prone to hypothermia. Typically, the animal will painlessly expire during the perfusion process, but if the animal survives or lingers, euthanasia should be administered promptly.

The actual perfusion apparatus can be a simple gravity fed apparatus or a pump-driven setup. Either works equally well. The perfusion apparatus is generally constructed of a flushing reservoir (container that holds the flushing solution) and a fixative reservoir (container that holds the fixative). Each reservoir needs to have tubing connected to it with shutoff valves in place close to the reservoir. Distal to each shutoff valve is a tubing that connects each of the reservoirs to a three-way valve or "Y" connector. Additional tubing then runs distally for a length that is convenient to perform the perfusion operations from the three-way valve or "Y" connector. An additional valve is generally placed in this final segment of tubing so as to control volume flow. The tubing terminates with a fitting that can accommodate a needle, trocar, or catheter, which will be inserted into the animal. For rodents, a 1 in. 16–20G needle is commonly used. For larger animals, an appropriately sized trocar is usually used.

For the gravity fed apparatus, the height of the reservoir should be approximately 110–127 cm above the level of the animal's heart or placement of the TIP. The reservoir valves are typically left in a wide open position. Initially, the main line will be filled with fixative solution, but then the fixative valve should be closed and the system flushed completely with flushing solution. The most commonly used flushing solution is phosphate buffered normal saline (PBS ± 1000 IU Heparin/1000 mL PBS). Perfusion fixative solutions can be (1) cacodylate buffer (with or without sucrose), (2) 4% paraformaldehyde, (3) 4% paraformaldehyde/1% glutaraldehyde (McDowell Trump's fixative), and (4) 2% paraformaldehyde/2.5% glutaraldehyde (modified Karnovsky's fixative).

Prior to the initiating perfusion, a flow rate must be set. Flow rates vary according to species: rat, 50 mL/min; mouse, 5 mL/min; dog (adult beagle), 400 mL/min; dog (adult hound), 500 mL/min; nonhuman primate, 350 mL/min; rabbit, 250 mL/min; sheep, 600 mL/min; and pig, 600 + mL/min depending upon the size. The times for perfusion vary also. Flushing should take place for 2–4 min, 2 min for rodents, and 4 min for nonrodents. Fixative should be allowed to perfuse for 8–10 min, generally 8 min for rodents and 10 min for nonrodents.

Once the animal is at an acceptable anesthetic plane (see previous) and the animal placed in dorsal recumbency, the sternum should be reflected cranially by cutting through the ribs on both sides of the sternum. The cutting should result in the creation of an opening that is sufficiently wide to permit ready access to the thoracic cavity and the heart. Alternatively, for dogs, the ribs themselves may be opened on the animal's side between ribs four and seven. The sternum is held in place cranially either manually or with a clamp. The pericardium is incised, allowing full exteriorization of the heart from the pericardial sac. Before flushing commences, if body position is important, the body should be appropriately positioned before the flow of fluids begins. The flow of flushing solution is initiated and the TIP inserted into an area just dorsal to the apex of the heart and into the left ventricle. The TIP is then advanced under the atrioventricular valve to the base of the aorta. The right auricle is incised and opened to allow the elimination of the returning flushing/fixative solution. Alternatively, if a closed perfusion is to be performed, a TIP as large as or ideally larger than the perfusion TIP is placed into either the right atrium or ventricle to allow easy unrestricted drainage of the blood and solutions. The TIP after placement should be clamped in place in the left ventricle. The diaphragm is generally incised to allow for more facile egress of the returning fluids draining from the right heart (if open procedure). After completion of the appropriate flushing interval, the solution is switched from flushing solution to fixative solution. Generally speaking, evidence of fixation should appear within 60 s of the initiation of flow of fixative solution. These signs could include muscle fasciculations, tail movement, generalized stiffening, generalized stretching, or other muscular activity. A failure to observe any of these signs indicates that the fixative is not entering the circulation and TIP placement needs to

be checked. After completion of the appropriate fixation interval, perfusion is terminated and the TIP withdrawn.

One needs to assess the quality of the perfusion when fluid administration is complete. A high-quality perfusion has occurred when the intended tissues all appear to be fixed and firm, most of the circulatory system is absent of blood, and the brain is more pale than that of a nonperfused animal. An acceptable, but not ideal, level of perfusion has occurred when there is evidence of some perfusion fixation, but the tissues are only slightly less malleable than those of a nonperfused animal, the brain is not firm, the brain is not pale, and/or portions of the carcass obviously did not perfuse at all (tissue firmness and color or pallor). Finally, an unacceptable level of perfusion fixation has occurred when there is basically no evidence of perfusion fixation in any tissues; tissues are soft, the brain is soft, the brain is pink, and all tissues resemble those of a nonperfused animal.

Photography

One final special procedure topic in necropsy and gross pathology is photography (Edwards, 1988; McGavin, 1988; Saikia et al., 2008; Stack et al., 2001; Weinberg, 1997). Someone once said that "…a single picture is worth a thousand words." That is probably true, regardless of how well written and composed a description might be. These days with digital technology and automatic cameras, the taking and handling of photographs is much simpler than it has been historically. The photographing of tissue and organ specimens can be highly useful for purposes of documentation. Photographs coupled with modern communication technology permit a distant observer to easily visualized images of gross lesions and even histological sections, enhancing the quality of any pathology-based discussions.

The Society of Toxicologic Pathology has published a very useful reference on the use of image data in pathology (Tuomari et al., 2007). In general, images that are used for the generation of data are considered to be raw data. Alternatively, images that are not used to generate actual data are not considered to be raw data. Under current technology, any image that is used for the purpose of generation of data, it becomes raw data at the moment in time of the generation of the actual data. Each image as it becomes raw data must be suitably documented indicating the person that generated it and time that the image was generated for data. Each photograph should always include some sort of reference marker indicating scale or size. These images that become raw data obviously must be archived. It should be obvious that relevant SOPs must be in place for the collection and use of images and the generation of data from such. Any equipment used must be appropriately qualified, tested, and validated. Relevant training records and procedures for those performing the procedures must be in place. Any GLP exceptions must be stated as deviations in the compliance statement portion of the study protocol. Images that are not used to generate raw data do not have to be archived, unless they are somehow referenced or used in the study report. One final point is that images in and of themselves cannot be used to override the study pathologist's written findings and observations at the time of actual necropsy nor overturn the study pathologist's report.

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Immunology Laboratory Endpoints for Assessment of Immunomodulators in Preclinical Studies and Case Studies Using Anti-CD3 mAb in Cynomolgus Monkey

Lynne LeSauteur, Simon Lavallee, and Marie-Soleil Piché

CONTENTS

ASSESSMENT OF IMMUNOMODULATION

Given the potential for manipulation of the immune system and impact on disease, immunomodulation is at the forefront of many drug discovery programs. Incorporating laboratory-based endpoints for assessment of efficacy and/or toxicity in preclinical studies for immunomodulators aids in the proper identification of drug candidates and is a regulatory requirement (ICHS6, 1997, 2012; ICHS6(R1), 2011; ICHS8, 2005). This chapter will outline immunology laboratory-based assays that are commonly included in preclinical studies to assess immunomodulation. An example of successful incorporation of immunology laboratory endpoints in a preclinical cynomolgus monkey efficacy study to monitor biomarkers of immune modulation and inflammation will be described to highlight study design, interpretation, and points to consider.

Immunomodulation describes the pharmacodynamic and efficacy evaluation of immunomodulators, which can range from overt toxicity to intended pharmacology. The immune system consists of cells in bone marrow, thymus, lymphatic system ducts and nodes, spleen, and blood that function to protect the body from foreign entities. The potential for adverse health effects in humans due to alterations in the immune system has been a matter of increasing scientific and public concern. Immunotoxicity encompasses any adverse effect on the structure or function of the immune system and can be classified into five effects: immunosuppression, immunostimulation, immunogenicity, autoimmunity, and hypersensitivity. There are numerous common useful immunology assays (immunoassays, the T-cell-dependent antibody response [TDAR], flow cytometry assays, and cell-based assays) to measure immunomodulation in many species (Table 20.1). These assays are chosen and incorporated into toxicology studies of the relevant species depending on the pharmacology, toxicology, availability of appropriate reagents, and validated assays. The ultimate goal for incorporating these assays is to allow for earlier robust drug safety and efficacy measurements.

With regard to evaluation of immunotoxicity in preclinical studies, the primary endpoints are standard hematology, clinical chemistry, gross pathology, organ weights, and histopathology of lymphoid organs. Secondary endpoints incorporated into preclinical toxicology studies of the appropriate species are driven by findings from standard toxicity studies, the pharmacological properties of the drug, the intended patient population, structural similarities to known immunomodulators, disposition of the drug in lymphoid organs, and clinical information including known immunomodulatory effects. Some of these secondary endpoints could include immunohistochemical evaluation of affected cell types, biomarker assessments, the use of flow cytometry to immunophenotype, and evaluation of specific cell subsets and cell function. Common functional assays include the natural killer (NK)-cell activity and macrophage function (oxidative burst and phagocytosis). In addition, because of the antigenic nature of protein therapeutics, immunogenicity testing and addressing the impact of antidrug antibodies on drug pharmacodynamics, pharmacokinetics, safety, and efficacy are standard requirements from regulatory agencies (EMEA 2008; FDA 2009, 2014).

Table 20.1   Common Immunology Laboratory Based Assays to Assess Immunomodulation

Assay Type	Matrix	Specie
Immunoassays		
Antibody detection	Serum	All species
Cytokines	Serum	Rat, mouse, primate
Chemokines	Serum	Rat, mouse, primate
Complement factors	Plasma	Primate
T cell dependent antibody response	Serum	Rat, mouse, primate, dog
Flow cytometry assay		
Multivariate surface and cytoplasmic immunophenotyping	Blood, tissue	All species (limited for dog)
Phenotyping of activation markers	Blood, tissue	All species (limited for dog)
Macrophage function	Blood	Primate
Receptor binding	Cells	All species
Intracellular cytokine staining	Blood, tissue	All species
Phosphorylation of proteins	Blood, tissue	All species
Cell based assays		
Neutralizing antibody assays	Serum	All species
Natural killer (NK) cell activity	PBMC, blood	Rat, primate
Cytokine release assays	PBMC, blood	All species
Ex vivo stimulation assays	PBMC, blood	All species

Note: All species refers to: rat, mouse, cynomolgus monkey and dog.

IMMUNOLOGY LABORATORY ENDPOINTS

Immunoassays

Immunoassays are commonly used to monitor protein biomarkers, including acute-phase proteins, complement split products, cytokines, chemokines, and antibodies (such as total IgG, IgM, and IgE). IgE can in particular be used to monitor hypersensitivity along with histamine quantitated. The incorporation of these biomarkers to monitor toxicity and efficacy within preclinical studies is increasing, although challenges exist regarding the translatability of biomarkers and the levels associated with toxicity, as has been reviewed recently for cytokine biomarkers (Tarrant 2010). However, biomarkers have proven to be useful to monitor many conditions such as inflammation.

C-Reactive Protein

The acute-phase response is a series of reactions initiated in response to a variety of inflammatory conditions and is characterized by leukocytosis, fever, alterations in the metabolism of organs, and changes in plasma concentration of numerous acute-phase proteins including fibrinogen, serum amyloid A, albumin, and C-reactive protein (CRP) (Marnell et al. 2005; Ponce 2008). The levels of CRP rise in response to inflammation and this protein plays a role in activating the classical complement cascade among other effector functions (Mortensen 2001). Thus, CRP is a useful biomarker of inflammation that can be quantitated using available immunoassays for cynomolgus monkeys.

Complement Proteins

Three biochemical pathways activate the complement system: the classical complement pathway that typically requires antibodies for activation, the alternate pathway that can be activated without the presence of antibodies, and the mannose-binding lectin pathway (Zipfel and Skerka 2009). All three pathways converge at the C3 protein level leading to the formation of the terminal complement complex (C5b-9). Activation of the complement system leads to cytolysis, chemotaxis, opsonization, immune clearance, and inflammation, as well as the identification of pathogens for phagocytosis. Spontaneous or continual activation of the complement system as a result of therapeutic exposure can produce anaphylactoids that can damage the host tissue. There is a series of immunoassays available to monitor various complement split products. Most commonly, Bb fragment, a marker of the alternative complement pathway; C3a, a marker of total complement hemolytic activity; and C5a are quantitated using immunoassays in cynomolgus monkey plasma samples.

Cytokines

Cytokines can be measured by singleplex or multiplex immunoassay in many matrices. Their measurement requires sensitive and specific assays and appropriate understanding of the kinetics of cytokine release and the associated pharmacology/toxicology related to the test article. In addition, assay variables and limitations need to be well understood. These immunoassays are validated based on a fit-for-purpose approach (Lee and Hall 2009). In vitro cytokine release assays have also increased in popularity due to the need to screen compounds for cytokine storm. A recent dramatic example of the cytokine storm phenomenon occurred in a first-in-human trial, where within hours of single-dose administration of the CD28 agonist IgG4 monoclonal antibody (mAb) TGN1412, a multitude of inflammatory cytokines were released leading to subsequent serious toxicities (Suntharalingam et al. 2006). However, with TGN1412, there were no substantial preclinical cytokine elevations (in serum or after in vitro stimulation) evident that would have predicted these clinical results (Stebbings et al. 2007), thus questioning the utility of preclinical studies to predict cytokine storm in humans. Since that time, it has become apparent that cross-linking of the TGN1412 antibody is required for the human PBMC to release cytokines (Findlay et al. 2010; Stebbings et al. 2007). Many different assay platforms and formats can be used to detect cytokine release (Dhir et al. 2012; Walker et al. 2010), and careful consideration should be applied for selection of the assays and first-in-human dosing strategies to mitigate risk (EMEA 2007). This highlights the need for appropriate assay setup and strong understanding of the test item pharmacology and cytokine release mechanisms involved. Ultimately, the utility of any protein biomarker measured by immunoassay depends on whether the detectable changes correlate with measurable in vivo toxicity and an understanding of the associated mechanism of action.

Immunogenicity Testing

Immunogenicity testing is a concern for peptide and protein therapeutics and is an important factor in study interpretation Therefore, immunogenicity testing needs to be considered for these drug classes and any drugs that are potentially haptenic, as well as polymer and oligonucleotide therapeutics. Virtually all therapeutic proteins and peptides elicit some level of antibody response. The preclinical and clinical consequences of antibody formation vary with the type and quantity of antibody present but may impact on drug pharmacokinetics, pharmacodynamics, efficacy, and

safety. Special consideration should be given to those products where there is a risk that the antibody response could affect any endogenous counterpart (as highlighted by the occurrence of pure red-cell aplasia and antierythropoietin [anti-EPO] antibodies in patients administered recombinant EPO [Casadevall et al. 2002]). Immunogenicity assessment has been performed by an enzyme-linked immunosorbent assay (ELISA); however, because of the concern for low-affinity binding antibodies and drug interference, other platforms, such as electrochemiluminescence (Meso Scale Discovery), are now used.

Method development and validation of immunogenicity assays are conducted in line with the most recent industry recommendations, regulatory guidelines, and white papers (Gupta et al. 2007; Koren et al. 2008; Mire-Sluis et al. 2004; Shankar et al. 2008). A flowchart of antidrug antibody analysis assays is shown in Figure 20.1. Assays for antibody detection can be grouped into two categories: binding assays and neutralizing antibody assays. Binding assays measure the ability of the antibodies present in the sera to bind to the antigen. They detect antibodies that are directed toward the molecule and thus detect both neutralizing and nonneutralizing antibodies. The second assay measures the ability of the sera to neutralize the biological activity of the drug (or antigen); it detects neutralizing antibodies. In general, antidrug antibody-binding assays consist of the detection of antidrug IgG and IgM antibodies. Other classes of antidrug antibodies such as IgE and IgA may be investigated on a case-by-case basis. If antidrug antibodies are detected, further characterization may be conducted including drug competition test (confirmatory assay), titration, and isotyping. There are three types of nAb assay formats: the cell-based bioassay, the non-cell-based competitive ligand-binding assay, and the enzyme-based assay. The cell-based bioassay is
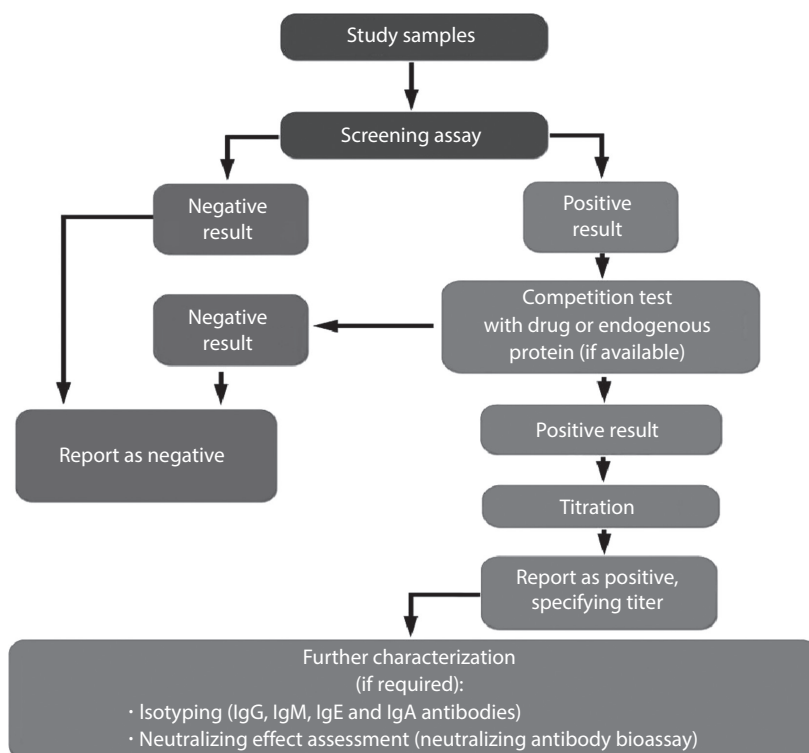
Figure 20.1   Flowchart of antidrug antibody analysis assays.

the preferred approach for measuring neutralizing antibodies, as it more closely mimics the neutralization of the drug's biological activity that can occur in vivo. To design such an assay, a cell line yielding measurable response to the drug needs to be available. If no suitable cell line can be generated, the non-cell-based competitive ligand assay (e.g., flow cytometry–binding assay) can be considered. Enzyme-based assays are needed when there is a concern about neutralizing antibodies impacting drug enzymatic activity. In order to evaluate the need and extent of immunogenicity testing in preclinical studies, one should consider the risk and whether PK and PD data are sufficient (Ponce et al. 2009).

T-Cell-Dependent Antibody Response

The TDAR assay is endorsed by regulatory agencies as a useful immune function test for evaluating the immunotoxic potential of a drug candidate as it involves antigen-presenting cells, B cells and T cells. It measures the ability of the host species to mount a specific antibody response to an antigen. Two standard antigens are routinely used to perform this type of assay: sheep red blood cell (SRBC) and keyhole limpet hemocyanin (KLH). In the primate, KLH is commonly used as the antigen and the TDAR assessment is incorporated into preclinical toxicology studies by monitoring anti-KLH antibodies via both the primary and secondary anti-KLH IgM and IgG responses if required. More recently, the KLH TDAR model has also been evaluated for its utility to monitor immunostimulation using suboptimal doses of KLH and monitoring augmentation of the TDAR response after drug administration (Piché et al. 2013). Although the TDAR is a useful assay to measure immunosuppression, on occasions host resistance assays (where animals can be challenged with various bacteria or viruses and the ability of the host to resist this infection after drug administration) are useful.

Flow Cytometry Assays

Flow cytometry is commonly used in studies to assess immunomodulation as changes in relative frequency and number of lymphoid and myeloid cells in the spleen, lymph nodes, bone marrow, and/or peripheral blood. In addition, functional assays such as NK-cell activity and macrophage function can be conducted using this technology.

There is a large panel of antibodies and assays available that can help characterize the impact on immune cells. For example, T-, B-, and NK-cell subsets in blood and lymphoid tissues as well as Th1 and Th2 responses, regulatory T cells, as well as dendritic cells can be enumerated and functional activity assessed using multiple biomarkers. When combining intracellular protein expression with surface marker expression in lymphoid cells, these assays become extremely powerful for assessing the effects of drugs on defined cell populations. Examples of assays that can be performed in lymphoid subsets include quantitation of various cell signalling molecules such as specific phosphoproteins. In addition, expressions of many adhesion molecules and growth receptors are altered on leukocytes during cell activation and differentiation. Many of these molecules are lineage associated and can be used to identify subsets as well as altered expression following exposure to drugs. Several examples of adhesion molecules that are up- or downregulated in response to immune activation or toxicants exist, the most common being CD25 (IL-2R), CD62L (L-selectin), and CD69 (C-type lectin). Other markers of cell activation and proliferation that are being increasingly utilized include various cytokine and chemokine receptors, particularly CCR7 for T cells, and proliferating cell antigens such as Ki67 protein. In addition, functional assays can be conducted by flow cytometry, such as phagocytosis and oxidative burst to evaluate drug impact on macrophages. Moreover, flow cytometry is being used to monitor cell injury and stress, receptor binding, and various biomarkers, in addition to the development of novel assays to monitor CD8 cell activation.

Cell-Based Assays

Various cell types of the immune system, including B, T, and NK cells, can be assessed using cell-based assays. When a drug targets cellular immunity, regulators may expect assays of cell proliferation, cytokine expression, antigen-specific T- or B-lymphocyte proliferation, and enzyme-linked immunospot (ELISpot). For example, T lymphocytes are responsible for cell-mediated immunity and are essential for the development and regulation of antibody responses, and their activity can be monitored by ex vivo proliferation assays. In addition, ELISPOT is also a useful assay format that can detect specific cytokines (such as interferon-gamma) at the single-cell level. NK cells are non–T and non–B lymphocytes that are an integral part of the first line of defense in innate or natural immunity. They contain granules with perforin and granzymes that facilitate spontaneous killing of target cells (tumor or virally infected cells). Of the assays used to assess the function of NK cells, the one most widely used is the chromium-release assay but flow cytometry–based assays to assess NK-cell functions have also been developed. In addition, basophil function assays are being more widely used to monitor hypersensitivity reactions.

ANTI-CD3 mAb PRECLINICAL EFFICACY STUDY

Cytokine release syndrome is a potential adverse effect attributed to cellular release of pro-inflammatory cytokines that occurs on initial and sometimes subsequent intravenous infusion of some types of protein biotherapeutics as exemplified by TGN1412 (Suntharalingam et al. 2006). For this reason, generalized systemic inflammation, whether caused by some biologics due to target activation as an off-target secondary event or indirectly as a result of tissue damage, has become a growing concern in the pharmaceutical industry. Measurement of biomarkers of inflammation has been shown to be a useful component of nonclinical testing approaches to identify compounds that have the potential to modulate the immune system.

In this study, an anti-CD3 mAb (clone FN18, Cell Sciences, mouse IgG1), binding to the TCR–CD3 complex on the surface of circulating monkey T cells, was used in cynomolgus monkey. It shares certain properties with its antihuman counterpart OKT3 that binds human and chimpanzee CD3, but does not bind cynomolgus monkey CD3 (Hart et al. 2004). It induces T-cell activation and has immunosuppressive function. OKT3 was the first mAb licensed as an immunosuppressive agent in transplantation, type 1 diabetes, and psoriasis (Chatenoud 2003; Utset et al. 2002), although it is associated with cytokine release syndrome (Roayaie et al. 2000) and complement activation (Raasveld et al. 1993). To our knowledge this is the first study describing FN18 administration to primates and translatability of serum cytokine levels in vivo to in vitro cytokine release in the same primates.

This study was undertaken to characterize the range and dynamics of impact of the administration of the anti-CD3 mAb FN18 on blood lymphocytes and inflammatory responses by monitoring cytokines, CRP, and complement factors in cynomolgus monkey. The cytokine serum levels obtained in vivo postadministration were also compared to those obtained following ex vivo challenge with the same antibody. The need to conduct this study was driven by the TeGenero incident to gain a better understanding of the ability of these assays to monitor inflammation with an antibody known to induce cytokine release in primate and to evaluate the usefulness of the in vitro whole blood cytokine release as a predictive tool for in vivo cytokine release. Thus, this study did not encompass all the standard preclinical toxicology evaluations required for mAbs, but focused on the common immunology assays incorporated into preclinical primate studies to monitor inflammatory responses.

Material and Methods

In Vivo Stimulation with Anti-CD3 mAb

Mouse anti-monkey-CD3 IgG$_1$ monoclonal antibody (anti-CD3 mAb, clone FN18, Cell Sciences) was formulated with 0.9% sterile saline. The study design is shown in Figure 20.2. Cynomolgus monkeys (1 male and 1 female per group) were injected with FN18 at dose levels of 0, 10, 50, and 100 μg/kg. Blood samples were collected into K$_2$ EDTA tubes for cytokine evaluation and into serum separator tubes (SST) tubes for CRP and C3a evaluation once prior to Day 1 of dosing, then at 30, 60, and 90 min and 3, 6, 12, 24, and 48 hours postinjection of the challenge article. Blood samples were collected into K$_2$ EDTA tubes for immunophenotyping once prior to Day 1 of dosing and then, 24, 48, and 144 hours postinjection of FN18. All animal studies were done in accordance with approved Institutional Animal Care and Use Committee protocols at Charles River.

Immunophenotyping of Lymphocyte Subsets in Blood

Immunophenotyping was performed by adding the following antibodies to 100 μL of whole blood.

Tube Number	Antibodies (BD Bioscience)
1	Dulbecco's phosphate-buffered saline (D-PBS) (Gibco)
2	Mouse IgG$_{1\lambda}$ antihuman CD3-PE
	Mouse IgG$_{1\kappa}$ antihuman CD4-APC
	Mouse IgG$_{1\kappa}$ antihuman CD8-FITC
	Mouse IgG$_{1\kappa}$ anti-NHP CD45-PerCP
3	Mouse IgG$_{1\lambda}$ antihuman CD3-PE
	Mouse IgG$_{1\kappa}$ antihuman CD16-FITC
	Mouse IgG$_{1\kappa}$ antihuman CD20-APC
	Mouse IgG$_{1\kappa}$ anti-NHP CD45-PerCP

Following incubation of the whole blood with D-PBS or antibodies, erythrocytes were lysed with FACS Lysing Solution (BD Biosciences), and the cells were washed twice with D-PBS containing sodium azide (Sigma–Aldrich) and resuspended in FACS Flow (BD Biosciences) containing formalin (Cochimbec). Stained cells were analyzed by flow cytometry on a FACSCalibur, and the analysis was performed using the CellQuest Pro software version 4.0.2 (Becton Dickinson).
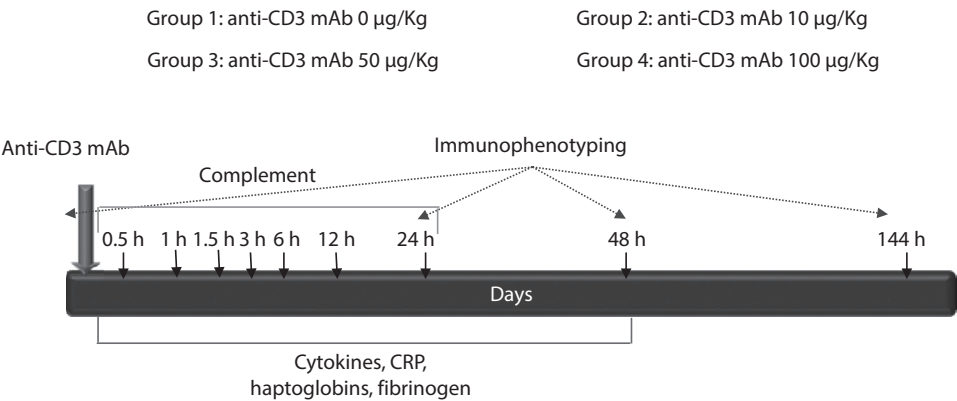
Figure 20.2 FN18 study design.

Absolute lymphocyte counts were determined using the Trucount tubes (BD Biosciences). Whole blood was incubated in a TruCount tube, with Mouse $IgG_1\kappa$ anti-NHP CD45-PerCP (BD Biosciences). Erythrocytes were then lysed with FACS Lysing Solution (BD Biosciences). Stained cells were analyzed by flow cytometry on a FACSCalibur, and analysis was performed using the CellQuest Pro software (Becton Dickinson). Peripheral blood immunophenotyping results were reported as absolute lymphocyte counts using BD TruCount™ tubes and reported as CD45+ lymphocytes per μL of whole blood. In addition, relative proportions and absolute numbers of total T lymphocytes (CD45+/CD3+), helper T lymphocytes (CD45+/CD3+/CD4+), cytotoxic T lymphocytes (CD45+/CD3+/CD8+), B lymphocytes (CD45+/CD3−/CD20+), and NK lymphocytes (CD45+/CD3−/CD16+) were reported.

CRP and the Acute-Phase Response

CRP concentrations in monkey serum were quantitated using a Monkey CRP ELISA kit (Life Diagnostics). Blood samples for CRP were allowed to clot at room temperature for at least 30 min and then centrifuged at $1200 \times g$ for 10 min in a centrifuge set to maintain 4°C. Serum samples were frozen immediately over solid carbon dioxide (dry ice) and subsequently stored in a freezer set to maintain −80°C until analysis.

Fibrinogen

Fibrinogen concentration was determined using the HemasIL® PT-Fibrinogen assay. Blood samples for fibrinogen were collected in sodium citrate tubes and were then centrifuged at $2400 \times g$ for 15 min in a centrifuge set to maintain 4°C. Plasma samples were frozen immediately over solid carbon dioxide (dry ice) and subsequently stored in a freezer set to maintain −80°C until analysis.

Haptoglobin

Haptoglobin concentrations were determined by colorimetric assay using the Phase® Range kit (Tridelta Development Ltd) that adapted an automated P800 Modular Analytics analyzer (Roche/Hitachi). Blood samples were collected in SST tubes and were allowed to clot at room temperature for at least 30 min and were then centrifuged at $1400 \times g$ for 15 min in a centrifuge set to maintain 4°C. Serum samples were frozen immediately over solid carbon dioxide (dry ice) and subsequently stored in a freezer set to maintain −80°C until analysis.

Complement Split Products in Plasma

C3a and Bb fragment concentrations in monkey serum were determined using ELISA kits (Quidel). Blood samples for Bb and C3a evaluation were centrifuged at $1200 \times g$ for 10 min in a centrifuge set to maintain 4°C, and the plasma samples were frozen immediately over solid carbon dioxide (dry ice) and subsequently stored in a freezer set to maintain −80°C.

Serum Cytokine Measurements

IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12/23 (p40), IL-13, IL-17, IFNγ, TNF-α, G-CSF, and GM-CSF concentrations in monkey plasma were determined by a bead-based multiplexed assay(Luminex®) using the Non-Human Primate Cytokine Kit (EMD Millipore Corporation). Blood samples for cytokines evaluation were centrifuged at $1200 \times g$ for 10 min in a centrifuge set to maintain 4°C, and the plasma samples were frozen immediately over solid carbon dioxide (dry ice) and subsequently stored in a freezer set to maintain −80°C until analysis.

In Vitro Cytokine Release Assays

Whole blood for stimulation was collected pretreatment into heparin tubes and was placed on wet ice until processing. For each monkey of the study, the anti-CD3 mAb concentration to be used for whole blood stimulation was evaluated based on body weight and estimated blood volume to reflect plasmatic concentration after injection.

Anti-CD3 mAb was added (at dose equivalents) and incubated with whole blood in solution (i.e., was not dry or wet coated) for 24 hours. The same panel used for serum cytokine evaluation was employed; IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12/23 (p40), IL-13, IL-17, IFNγ, TNF-α, G-CSF, and GM-CSF were measured in cell culture supernatant using the same Non-Human Primate Cytokine Kit (EMD Millipore Corporation).

Results and Discussion

Immunophenotyping of Lymphocyte Subsets in Blood

The primary objective for the immunophenotyping was to monitor the timecourse of reduction of T cells due to anti-CD3 mAb administration by flow cytometry. As expected, a marked decrease was measured in the total T, helper T, and cytotoxic T lymphocytes, which was also reflected in a drop of the total lymphocyte counts in all animals 24 hours following dosing when compared to the pretreatment results (Figure 20.3) with the exception of the female administered 100 μg/kg of anti-CD3 mAb. This was the only animal in which no decrease in T-cell populations was observed. In addition, little or no cytokine response was observed of this animal. The sensitivity of this female to the anti-CD3 mAb used may be lower than the other animals due to CD3 polymorphism (Liu et al. 2007). All analyzed T-cell populations returned to approximately baseline or higher levels by 144 hours postinjection. There was no impact on B-cell or NK-cell populations (data not shown).

CRP and the Acute-Phase Response

CRP increases were observed, peaking between 12 and 24 hours postinjection in all animals. CRP levels were back to approximately baseline 48 hours postadministration in all animals except for the male administered with 50 μg/kg of anti-CD3 mAb (Figure 20.4). The high sustainable CRP response observed in this animal correlated with elevated cytokine levels, indicating that the inflammatory response was stronger in this animal. Lack of dose dependence in CRP levels was apparent in both males and females as induction of CRP was greater at 10 and 50 μg/kg than 100 μg/kg.

Haptoglobin and fibrinogen acute-phase proteins were also measured at different timepoints following dosing. Haptoglobin increases were observed at all doses in males and females except in the female dosed at 100 μg/kg (Figure 20.5). Increases in fibrinogen, greater than what was observed in control animals, were observed in males dosed at 50 and 100 μg/kg. In females, no significant increases in fibrinogen levels were observed at all dose groups (Figure 20.6). The fact that no increases in haptoglobin and fibrinogen were seen in the 100 μg/kg female may be due in part to the potential lower sensitivity of this animal to the anti-CD3. Mild adverse reactions including redness of skin, vomiting, and loss of appetite were observed in all animals from all dose groups. However, all clinical signs were no longer observed 1 week after dosing.

Complement Factors in Plasma

In the male given 50 μg/kg of anti-CD3 mAb, increases in C3a concentrations were observed, peaking 6 hours following dosing and remaining higher than baseline levels 24 hours postinjection.
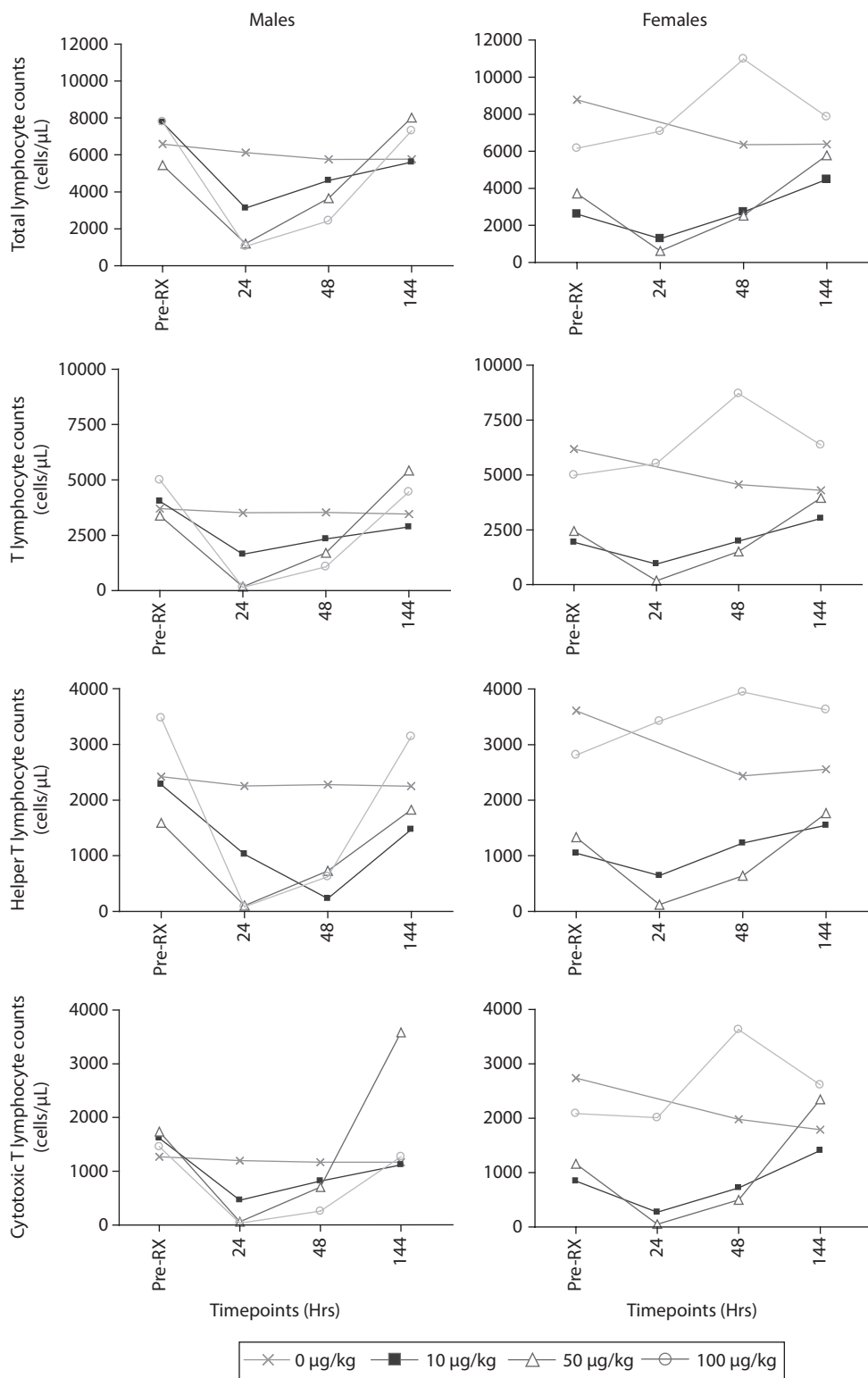
Figure 20.3 Lymphocyte counts in cynomolgus monkeys following FN18 administration.
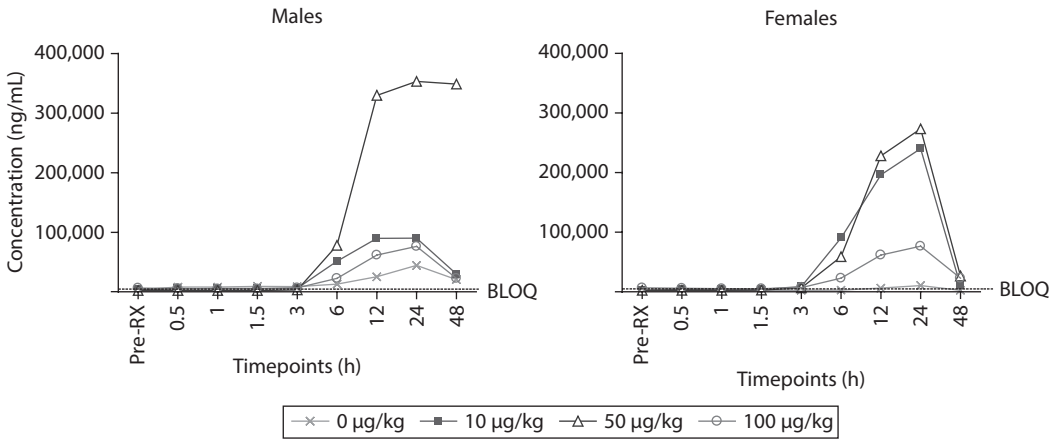
Figure 20.4 CRP response in cynomolgus monkeys following FN18 administration.
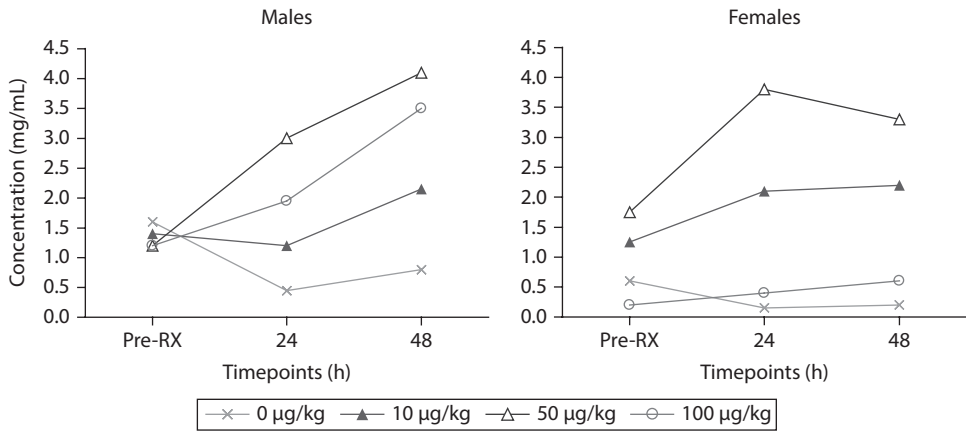
Figure 20.5 Haptoglobin response in cynomolgus monkeys following FN18 administration.
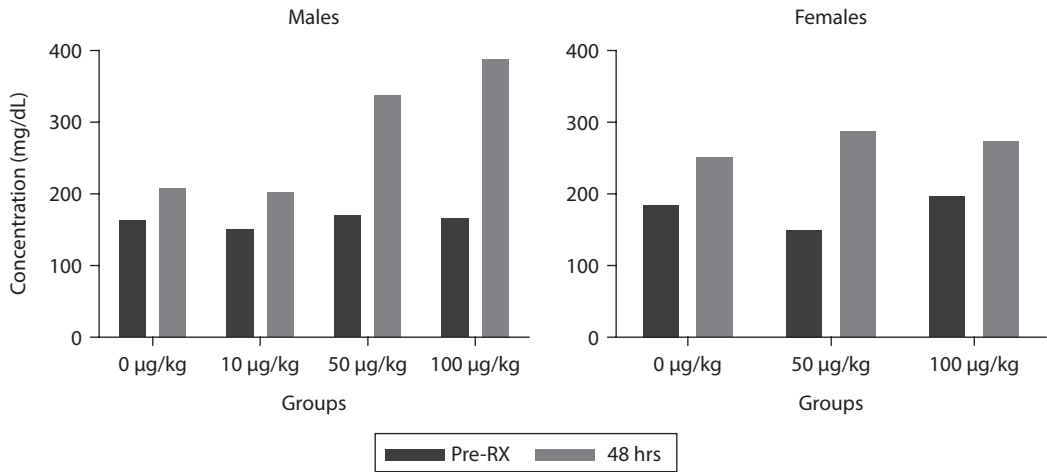
Figure 20.6 Fibrinogen response in cynomolgus monkeys following FN18 administration.

Figure 20.7 Complement response in cynomolgus monkeys following FN18 administration.

This increase in C3a correlated with the highest and most sustainable levels of CRP. A slight increase in C3a was apparent 6 hours postdose in the 10 and 50 μg/kg females. However, these increases were mild and within the normal range of values obtained in the control male. Therefore, they could not be attributed to FN18 administration. The same pattern was observed for Bb fragment, where all values obtained were within the range of control animals (Figure 20.7).

Serum Cytokine Measurements

Both proinflammatory cytokines (IL-2, TNF-α, INF-γ, IL-12, IL-5, IL-6, G-CSF) and anti-inflammatory cytokines (IL-10 and IL-RA [receptor antagonist]) were monitored. Anti-CD3 mAb administration led to a rapid and temporary release of proinflammatory cytokines IL-2, TNF-α, INF-γ, IL-12, IL-5, IL-6, and G-CSF that were predominant in the male and female administered with the 50 μg/mL dose (Figure 20.8a and b). While there was some cytokine release observed in the male dosed at 100 μg/kg (IL-2, IL-12, IL-5, IL-6, and G-CSF) and IL-2 release in the female dosed at 10 μg/kg, these responses were not as elevated. Increases in anti-inflammatory cytokines IL-1RA and IL-10 were observed for most treated animals. In the males given 10 and 100 μg/kg, a moderate to large early release of IL-10 was observed and was accompanied by a lower cytokine inflammatory response (IL-2, IL-12, IL-5, IL-6, and G-CSF) for both animals, when compared to the male dosed at 50 μg/kg. All cytokine values were back to
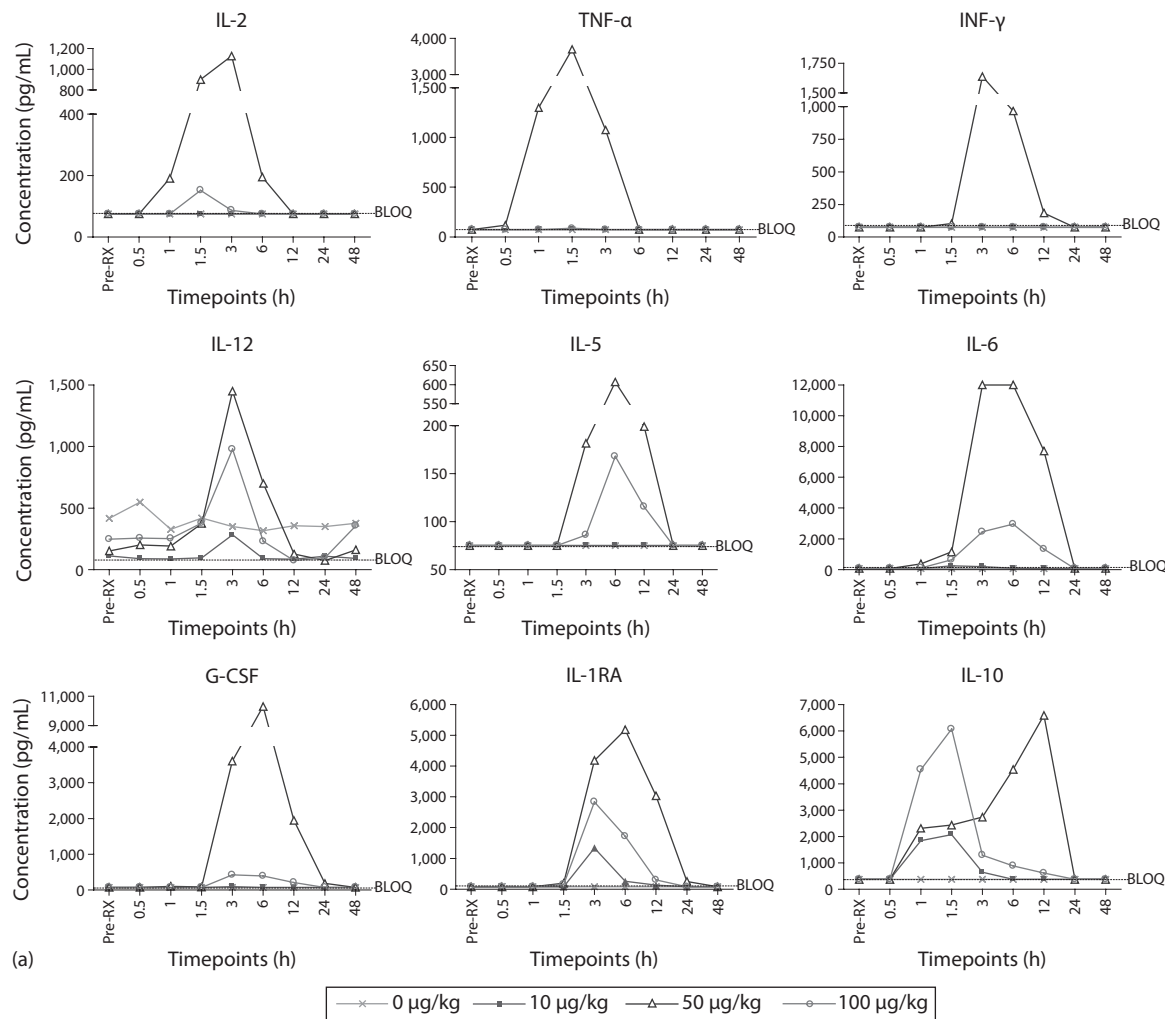
Figure 20.8 Cytokine responses in (a) male. (*Continued*)
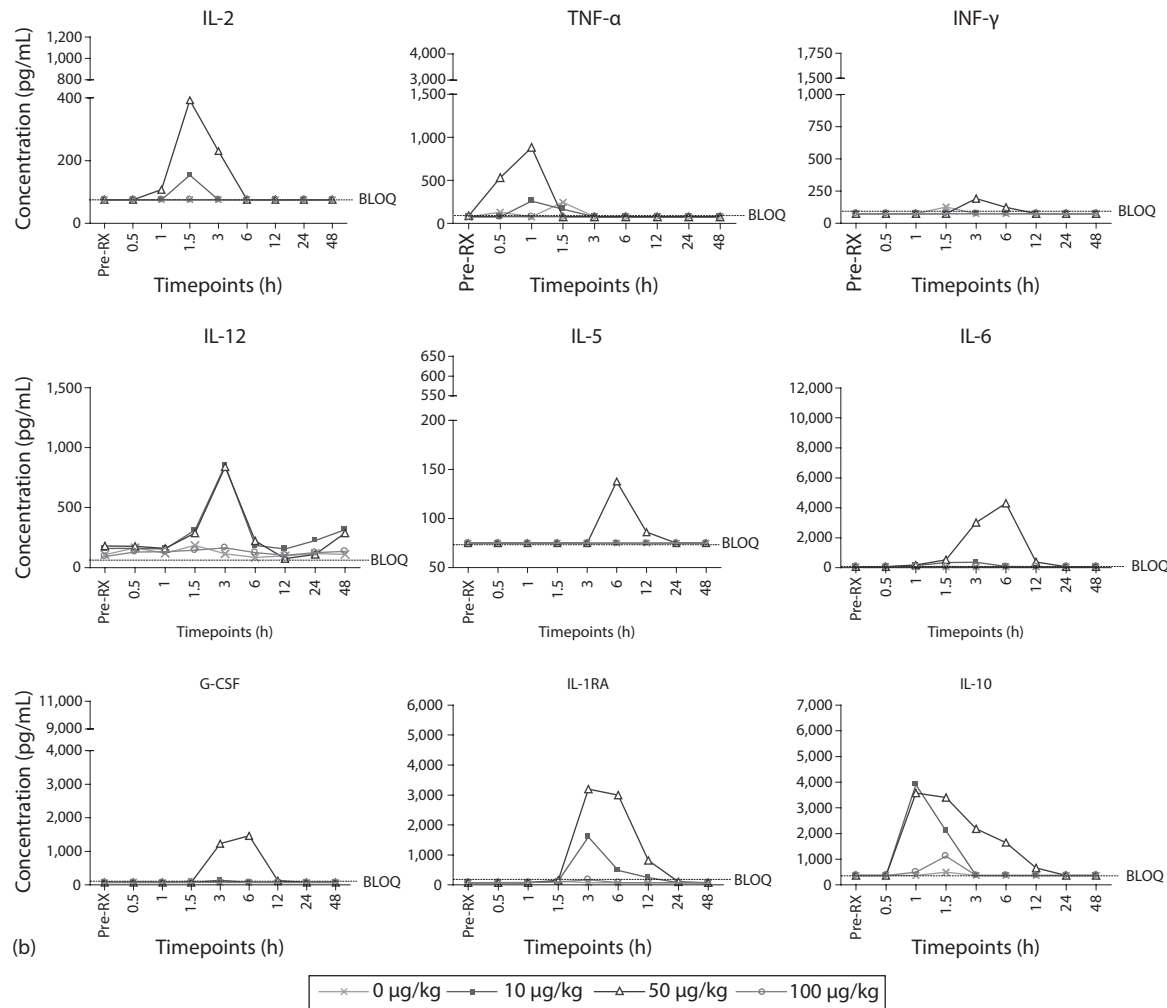
Figure 20.8 (*Continued*) Cytokine responses in (b) cynomolgus monkeys following FN18 administration.

approximately baseline levels 24 hours postadministration. Values below or close to the lower limit of quantification were obtained for IL-1β, IL-4, IL-13, IL-17, and GM-CSF at all timepoints tested (data not shown). In general, the lack of response in the 100 µg/kg female in terms of cytokine and acute-phase protein increases correlated with the lack of T cells decrease measured by immunophenotyping.

In Vitro Cytokine Release Assays

The in vitro whole blood stimulation with FN18 led to the release of IFN-γ and TNF-α and a slight increase in IL-1RA in the female administered 10 µg/kg, whereas increases in these three cytokines and IL-2 were observed in the male dosed at 50 µg/kg (Figure 20.9). All the other cytokine levels in any other group were below the lower limit of quantification (data not shown).

The pattern of cytokines released after in vitro stimulation was not identical to the pattern detected in the serum of animals treated in vivo with the anti-CD3 mAb. The cytokine profile and intensity of in vitro cytokines detected was relatively moderate when compared to the cytokines detected in serum. In males, the only animal that released detectable levels of cytokines in vitro was the one dosed with 50 µg/kg of FN18 and the one presenting the highest serum cytokine levels in vivo. In females, however, the intensity of cytokine release in vivo did not correlate with cytokine release in vitro. No in vitro cytokine release was observed in the 50 µg/kg female, which generally presented the highest levels of cytokines in vivo, although these levels were lower than those observed in the male from the same dose group. In vitro cytokine release was only observed in the 10 µg/kg female, presenting moderate to low levels of cytokines in vivo. The differential responses in vitro and in vivo may be explained by the absence of lymphoid tissues in the in vitro system. Alternatively, the timing of the anti-CD3 mAb stimulation may have also contributed to the results obtained. Indeed, the kinetics of response was shown to be important in the serum cytokine detection, in which increases in cytokine production were observed as soon as 1 hour following injection. Presentation of the anti-CD3 mAb during the experiment might also be a factor in the poor response in the in vitro release assay. Dry or wet coating of the antibody, with appropriate orientation and/or cross-linking of the monoclonal antibody, might have yielded a stronger response. This emphasizes the need for appropriate understanding of the limitations of the assay design, the timepoints of sample collection, and the time of stimulation in vitro.
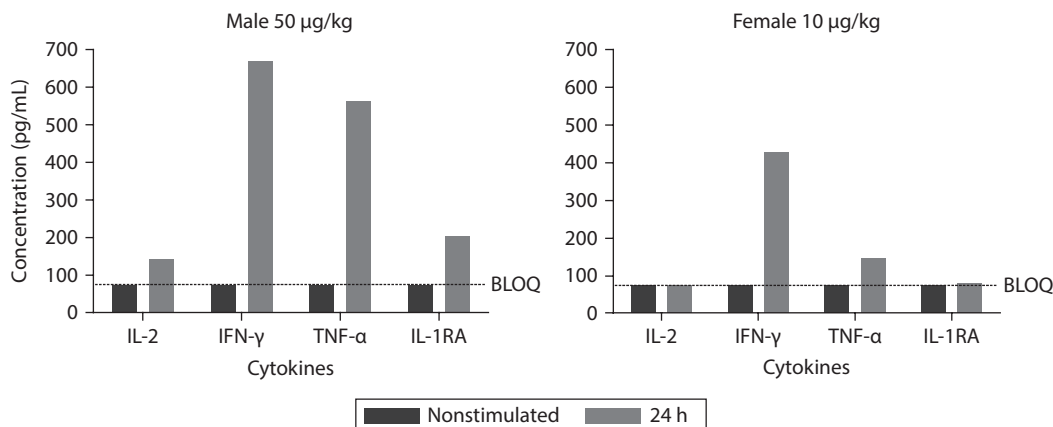
Figure 20.9   Ex vivo cytokine release following stimulation with FN18.

SUMMARY AND FUTURE DIRECTIONS

We demonstrated in this study with the anti-CD3 mAb FN18 that analytical methods, immuno-phenotyping, and biomarker assays (acute-phase proteins, complement split products, ex vivo and in vitro cytokine release assays) are suitably sensitive to detect changes in inflammatory biomarker status. Like OKT3 in humans, FN18 depleted T cells in cynomolgus monkeys and was associated with increase in biomarkers of inflammation including cytokines, acute-phase proteins, and C3a. The levels of induction of different inflammatory mediators in the case study presented would be a concern should the monoclonal antibody be further used in the clinic. Additional immunology assessments (in vitro and in vivo) and better characterization of the inflammatory response and its effects would need to be performed. However, from the data obtained in the in vitro system, it is clear that translatability of these assays remains a challenge; the in vitro assay did not accurately predict the level of cytokine release in vivo and the pattern of release differed in both systems. Therefore, in this case, in vitro cytokine release assays may be predictive of intensity but not nec-essarily of occurrence of the cytokine release. This further highlights challenges in interpretation and study design. Although many immunology-based assays are available that can be used in pre-clinical studies to monitor immunotoxicity and immunomodulation, additional needs include better assays to monitor hypersensitivity and autoimmunity, genomics-based assessments, and immuno-pharmacology assays as well as increased incorporation and translatability of biomarker assays in preclinical studies.

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Current Commercial Sources of Laboratory Animals by Species

Source	Rat	Mouse	Hamster	Gerbil	Ferret	Dog	Rabbit	Guinea Pig	Nonhuman Primate	Miniswine	Zebrafish	Transgenic Rat	Transgenic Mouse	Gene Knockout Rat	Gene Knockout Mouse	Gene Knockout Rabbit	Amphibians
Alpha Genesis, Inc.									X								
Animal Technologies Ltd.	X																
Antech, Inc.						X											
B&K Universal Ltd.		X			X	X	X	X					X				
Brown Family Enterprises, LLC (formerly Gemini Research)						X											
Buckshire Corp.									X								
Charles River Laboratories	X	X	X	X			X	X	X			X	X				
Covance Research Products			X	X		X	X		X								
Cytogen Research and Development, Inc.		X															
Ekkwill Waterlife Resources																	X
Ellegaard Göttingen Minipigs, A/S										X							
Elm Hill Breeding Labs, Inc.							X										
Harlan Laboratories, Inc.	X	X	X		X	X	X				X	X					
Hilltop Lab Animals	X	X						X									
Isoquimen, SL					X	X	X										
Kralek Farms, Inc.							X										
Liberty Research						X											
Lone Star Laboratory Swine										X							
Marshall BioResources						X	X			X							
Millbrook Breeding Lab							X										
NASCO International										X							X
Oak Hill Genetics						X											

(Continued)

Current Commercial Sources of Laboratory Animals by Species

Source	Species																
	Rat	Mouse	Hamster	Gerbil	Ferret	Dog	Rabbit	Guinea Pig	Nonhuman Primate	Miniswine	Zebrafish	Transgenic Rat	Transgenic Mouse	Gene Knockout Rat	Gene Knockout Mouse	Gene Knockout Rabbit	Amphibians
PrimGen Inc.									X								
Primate Products									X								
Ridglan Farms						X											
Robinson Services, Inc. (RSI)							X										
S & S Farms										X							
SAGE®Labs (formerly Ace Animals Inc.)	X	X										X	X	X	X	X	
Simonsen Laboratories, BioSourceOne Company	X	X	X														
Sinclair Bio-Resources, LLC										X							
SNBL USA									X								
Taconic	X	X										X	X	X	X		
The Jackson Laboratory		X											X				
Triple F Farms					X												
Western Oregon Rabbit Co.							X										
Worldwide Primates, Inc.									X								
Zebrafish International Resource Center (ZIRC)											X						

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