THE SCIENCE OF MEAT QUALITY

Edited by
Chris R. Kerth

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Edited by
CHRIS R. KERTH, PhD
Animal Science Department
Texas A&M University, USA

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Editorial Offices:
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The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, UK
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Contributors

Jason K. Apple	Department of Animal Science, University of Arkansas, Fayetteville, Arkansas
Christy L. Bratcher	Department of Animal Sciences, College of Agriculture, Auburn University, Auburn, Alabama
Kirk W. Braden	Department of Agriculture, Angelo State University, San Angelo, Texas
Terry Brandebourg	Department of Animal Sciences, College of Agriculture, Auburn University, Auburn, Alabama
Ryan Cox	Department of Animal Science, University of Minnesota, St. Paul, Minnesota
Patricia C. Curtis	Department of Poultry Science, College of Agriculture, Auburn University, Auburn, Alabama
Michael A. Davis	Baker County Extension Director, University of Florida, Macclenny, Florida
Michael Dikeman	Department of Animal Sciences and Industry, Kansas State University, Manhattan, Kansas
Chris R. Kerth	Department of Animal Science, College of Agriculture and Life Sciences, Texas A&M University, College Station, Texas
Richard Mancini	Department of Animal Sciences, College of Agriculture and Natural Resources, University of Connecticut, Storrs, Connecticut
Clinton Rowe	Food Scientist, ConAgra Foods, Omaha, Nebraska
Kyle Willian	Department of Chemistry, Tuskegee University, Tuskegee, Alabama

1 Growth of Muscle from the Myoblast to Whole Muscle

Terry Brandebourg

Introduction

Better understanding of the growth and development of skeletal muscle, and to a lesser extent, adipose tissue, is an important endeavor in meat science. This goal is driven by the need for the meat industry to consistently satisfy consumer demand for nutritious, high-quality, lean products in as efficient a manner as possible. Importantly, meat products are primarily derived from the skeletal muscle and associated fat of livestock.

Muscle growth, composition, and metabolism are integrally linked to meat quality through effects on yield, tenderness, and color. Typically, meat-producing animals are grown until an optimal balance between muscle mass and fattening is achieved. Upon slaughter, livestock carcasses are dressed leaving only the bones and edible muscles. Dressed carcasses are then aged in a temperature-controlled environment where biochemical processes such as glycolysis and protein degradation contribute to optimal meat quality.

Undoubtedly, advances in our understanding of factors that regulate the growth and development of muscle and the conversion of whole muscle to meat will lead to strategies that enhance meat quality. With these goals in view, this chapter will focus upon the growth of muscle from the myoblast precursor to whole muscle and upon cell culture techniques that allow these processes to be studied.

Overview of Skeletal Muscle Development

The growth of skeletal muscle can be meaningfully divided into stages by key developmental milestones. Landmarks such as conception, the maturation of the embryo (spanning the eight-cell stage through implantation), parturition, and finally, postnatal growth largely frame periods where specific mechanisms of growth contribute uniquely to muscle development. Thus, such landmarks represent useful points of reference that form a roadmap for better understanding of skeletal muscle development.

Viewed through this paradigm, skeletal muscle development can be divided roughly into two general phases of growth by parturition. Prenatal muscle development occurs primarily through increases in muscle fiber number (hyperplasia). Whereas postnatal growth of muscle is accomplished by increases in the size of preexisting muscle fibers (hypertrophy). In absence of injury, fiber number is essentially maintained during this period as very little new muscle fiber growth occurs after birth.

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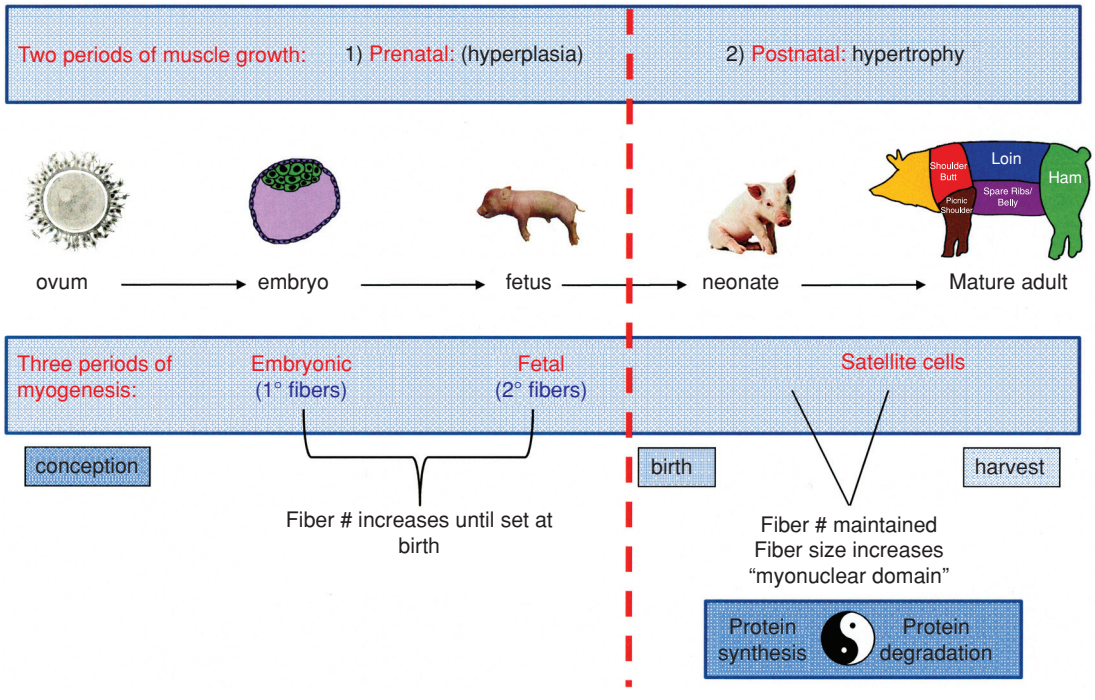

Figure 1.1 Roadmap for better understanding the skeletal muscle development based upon key developmental milestones. Such landmarks break development into discreet periods that are defined by unique mechanisms of muscle growth.

Alternatively, skeletal muscle development can be broken temporally into three phases of myogenesis. During early gestation, fiber number is increased via embryonic myogenesis (primary fiber formation). A second wave of fetal myogenesis (secondary fiber formation) then occurs during mid- to late-gestation. These two waves of prenatal myogenesis essentially dictate muscle fiber number in the adult. A third wave, satellite cell-related myogenesis mediates the postnatal increase in muscle fiber size that occurs in growing animals. Satellite cell fusion is also responsible for the maintenance of fiber number in the adult by facilitating the regeneration of damaged muscle fibers. Thus, an understanding of the regulation of myogenesis can largely inform all stages of skeletal muscle development (Fig. 1.1).

Types of Muscle

Three types of muscles can be distinguished structurally and physiologically in livestock. Smooth muscle is found in the walls of blood vessels, the lining of the gastrointestinal tract, uterine walls, and walls of respiratory passages. This type of muscle is innervated by the autonomic nervous system, thus its contraction is characterized by slow, but sustained contractile velocity that occurs without conscious thought. A second type of muscle, cardiac muscle, is innervated with an intrinsic nervous system unique to the heart that is specialized for generating highly controlled rhythmic contractions. Finally, skeletal muscle comprises the bulk of muscle in the body and its contraction is controlled by nerves emanating from the spinal cord. Importantly, skeletal muscle represents the primary source of meat from the carcass.

The unique and highly organized structure of skeletal muscle facilitates locomotion, a primary function of this muscle. Skeletal muscle appears striated due to the abundant expression of contractile apparatus proteins and, as discussed later, this muscle can appear reddish or whitish depending upon its fiber composition. Regardless of the anatomical location, skeletal muscles originate on a bone and terminate across the joint of another bone further away from the body's axis in such a way as to allow bones to rotate about the joint and move upon muscle contraction (Engel and Franzini-Armstrong, 2004). Muscles attach either via a tendon or upon a thin sheet of connective tissue (fascia).

Structure of Muscle

It is necessary to first appreciate the structural organization of skeletal muscle in order to understand why its development occurs as it does. The ultra-structure of the muscle cell and components of the contractile apparatus will be discussed in great detail in subsequent chapters. For now, we will focus upon the organization of the myofibril network and how this network interacts with the specialized membrane system of muscle fibers as these interactions are important for both prenatal and postnatal growth.

The basic structural unit of skeletal muscle is the muscle fiber (also termed muscle cell, myocyte, or myofiber). Muscle fibers are single, multinucleated cells that have peripherally located nuclei and a highly ordered and densely packed intracellular network of myofibrils, the organelle of contraction. Individual muscles are composed of a variable number of fibers that run parallel to one another. Individual fibers can be 10–100 μM in diameter and often reach many centimeters in length in the adult. Muscle fibers generally stretch from the origin to insertion in whole muscle though some individual muscle fibers terminate intrafascicularly (do not extend this entire length). Thus, intrafascicular fibers can confound efforts to quantify fiber number. Their size, multinucleated nature, specialized contractile apparatus, and unique membrane system distinguish muscle fibers from other animal cells (Fig. 1.2).

Myofibrils are specialized organelles that constitute the contractile apparatus of the muscle fiber. The basic unit of the myofibril is a repeating structure called the sarcomere. Each sarcomere comprises adjacent bundles of actin and myosin filaments which are capped on either end by a dense, proteinasceous Z-disc. Thin actin filaments extending from the Z-discs run in parallel to thick myosin filaments that appear to be centrally located in the sarcomere. Contraction occurs as these filaments interact and slide along one another thus drawing opposing Z-discs closer together.

In keeping with its unique structure, the muscle fiber is defined by a specialized, semi-permeable plasma membrane called the sarcolemma. The large number of channels and pores dispersed throughout the sarcolemma facilitate synaptic transmission and action potential propagation in response to acetylcholine release by motor neurons at neuromuscular junctions (Engel and Franzini-Armstrong, 2004). Thus, the sarcolemma is integral to proper contractile function in addition to performing the normal processes associated with a plasma membrane.

Immediately surrounding the sarcolemma is an extracellular matrix whose components are secreted by the muscle fiber proper. This thin layer of basal lamina and interwoven network of reticular fibers is directly attached to the sarcolemma and functions to maintain muscle integrity. Growth factors that bind to proteoglycans such as heparin sulfates in the basal lamina have been shown to regulate myogenesis and postnatal hypertrophy of muscle cells (Campbell and Stull, 2003). Furthermore, observed links between the basement membrane and sarcomeres of the myofibril suggest that the basal lamina may also function in contraction and myofibrillogenesis though the mechanisms are

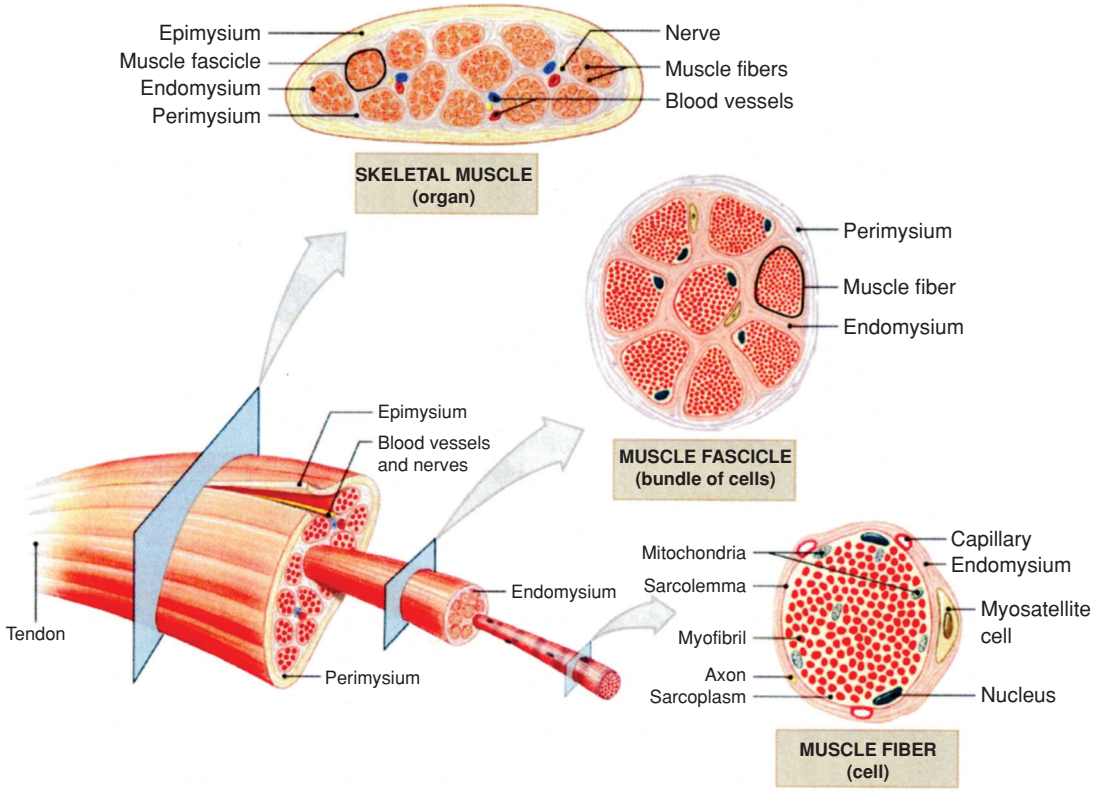

Figure 1.2 The organization of skeletal muscle. The muscle fiber is the basic structural unit of skeletal muscle. Fibers are further bundled into fascicles by the perimysium. Fascicles are then grouped into whole muscle by the epimysium. These connective tissue layers are highly organized and their function has implications for meat quality and muscle development. Modified from Wood et al. (2010).

poorly understood (Street, 1983; Danowski et al., 1992; Ervasti, 2003). Importantly, the basement membrane also facilitates connection of the muscle to tendons.

The endomysium, a final layer of connective tissue primarily composed of reticular fibers, encapsulates the muscle fiber proper and surrounding basement membrane. The endomysium is attached to the sarcolemma by multiple interactions with the basal lamina and functions as a sheath that allows capillaries, nerves, and lymphatics to reside in close proximity to the muscle fiber (Gerrard and Grant, 2003). Importantly, the endomysium is thought to influence meat tenderness (Swatland, 1975a, 1975b, 1975c; Lawrie, 1991).

Organization of Whole Muscle

Just as muscle fibers are multilayered structural units comprising of specialized membranes and connective tissue, whole muscle is organized by an elaborate, ordered system of connective tissue layers that can affect muscle function and meat quality. Among these connective tissue sheaths are the perimysium and the epimysium. The perimysium organizes individual fibers into bundles

called muscle fascicles. The number of individual fibers encased within the perimysium can vary. Furthermore, fascicular number and size varies with the function and anatomical location of the muscle. Importantly, intramuscular fat develops both within the perimysium and between bundles so it is possible that local signals emanating from the perimysium may affect the development of marbling through influencing either fat cell number or lipid filling. The epimysium encases multiple muscle fascicles effectively bundling them together to form the whole muscle. The epimysium is continuous around a muscle and is contiguous with the perimysium and endomysium ultimately thickening at junctions with tendons where muscle connects.

These individual layers intercalate to form a rich network that in addition to providing structural integrity also influences muscle growth both through direct and indirect effects. Physical links between the connective tissue layers and muscle fibers are thought to play important roles in regulating myofibrillogenesis and myogenesis (Menko and Boettiger, 1987; Rosen et al., 1992). Indirect effects can also be manifested by the ability of these layers to act as a reservoir for growth factors that are sequestered by proteoglycans in these rich networks (Timpl and Brown, 1996).

Although the muscle fiber is the basic structural unit of skeletal muscle, whole muscle is in fact heterogeneous with regard to cell types found within the tissue. Specialized mononucleated pre-muscle cells called satellite cells can reside between the endomysium and sarcolemma of the muscle fiber. Satellite cells play an important role in muscle hypertrophy serving as a local source of DNA that is essential to support postnatal muscle growth. Additionally, blood vessels, nerves, and lymphatics are interspersed between the perimysium and epimysium throughout the muscle. Motor neurons serve to activate a group of muscle cells which results in contraction of the fibers and thus facilitates movement. This relationship between a motor neuron and the downstream muscle fibers it stimulates is called a motor unit.

Fiber-Type Development

Heterogeneity is also a characteristic intrinsic to skeletal muscle fibers (Swatland, 1975a, 1975b, 1975c). Muscle fibers can be grouped as either slow oxidative, fast oxidative, or fast glycolytic based upon their speed of contraction and the predominant type of metabolism carried out by the fiber. Thus, muscle fibers can be distinguished by their functional properties through measuring the types of myosin heavy chain isoforms and mitochondrial oxidative enzymes expressed by the fiber. Fiber-type distribution within a muscle is related to the functional demands of the muscle, although generally within a given muscle, there will be a mosaic of fiber types with their distribution appearing more or less random (Swatland, 1975a).

Fiber-type distribution within a muscle can also affect the muscle's appearance. A muscle made up of predominantly oxidative muscle fibers will tend to be red in color, while a muscle consisting of predominantly glycolytic fibers will tend to appear white. This is because oxidative fibers have a high demand for oxygen to support their metabolism and thus contain a high myoglobin concentration. Myoglobin is a red pigment that functions to transfer oxygen from hemoglobin in the capillaries to the interior of the cell. However, there is a continuum in myosin heavy chain and mitochondrial enzyme expression in myofibers such that the rigid classification of fibers into three classes is not always possible.

Importantly, muscle fibers within a given muscle retain the ability to switch between types in response to environmental, physiological, or local signals thus allowing muscle to respond to changing demands but also complicating fiber typing. This is potentially significant because it suggests a mechanism whereby muscle mass can be altered independent of the need to alter fiber

number. Generally, glycolytic fibers are larger than oxidative fibers whose size may be limited by the need to diffuse oxygen efficiently. Glycolytic fibers also have a lower rate of protein turnover as well. Thus, white muscles are larger and more efficient. However, such fibers can be associated with poorer meat quality due to their color and metabolic properties. Therefore, the fiber-type composition of skeletal muscle as a whole can significantly impact meat quality. Genetic selection for rapid growth and leanness has generally resulted in a shift toward more glycolytic fibers in improved lines (Brocks et al., 2000).

The biology of fiber types and their impact on meat quality will be discussed in greater detail in later chapters but for now it is important to realize how the various fiber types arise during development. Primary muscle fibers initially form in the embryo and serve as scaffolds for secondary fiber formation. These initial fibers uniformly contain slow myosins regardless of the eventual presumptive muscle type they will give rise to in the fetus. As secondary myotubes form on the primary fiber scaffold, they become mixed in their myosin content. Although the etiology of fiber-type distribution in muscles of the developing fetus is still poorly understood, it is thought that innervations of the developing muscle by motor neurons dictates fiber type through orchestrating divergent calcium profiles in motor units (Beermann and Cassens, 1977a, 1977b; Beermann et al., 1977, 1978). Motor neurons excite skeletal muscle fibers by releasing the neurotransmitter, acetylcholine, into neuromuscular junctions. This in turn stimulates calcium release from the sarcoplasmic reticulum resulting in activation of the contractile apparatus. Chronic motor stimulation promotes a persistent, though low calcium level in the fiber that signals constant contraction, a phenotype consistent with oxidative fibers. On the other hand, intermittent stimulation signals spikes in intracellular concentrations of calcium resulting in rapid contraction, a phenotype characteristic of glycolytic fibers. The regulation of fiber type is currently an intense field of study given that manipulating the distribution of fiber types within muscle tissue has great potential to impact growth efficiency and quality.

Hyperplasia (Prenatal Muscle Development)

Overview

Skeletal muscle development is characterized by marked hyperplasia during gestation as muscle fiber number increases dramatically until birth. This increase in fiber number represents the primary mechanism of muscle growth during this period. Myogenesis is defined as the generation of muscle cell precursors and the subsequent fusion of such precursors to either form new fibers or to contribute DNA to existing fibers. As such, myogenesis drives the hyperplastic growth of prenatal muscle development. This complex process encompasses the recruitment of totipotent embryonic stem cells to the mesodermal lineage (determination) and the subsequent formation and maturation of muscle fibers. Importantly, myogenesis also extends beyond birth to include the postnatal addition of muscle cell precursor nuclei to mature fibers. Based upon this definition, skeletal muscle development can be broken temporally into three phases of myogenesis where fiber number is dictated by the extent of embryonic and fetal myogenesis and fiber size is determined by the degree of postnatal satellite cell fusion.

By influencing the number and type of myofibers that are present within whole muscle, factors that regulate myogenesis ultimately determine the potential mass of adult muscle tissue. Therefore, it is important to understand where muscle cell precursors originate and how they become functioning muscle fibers.

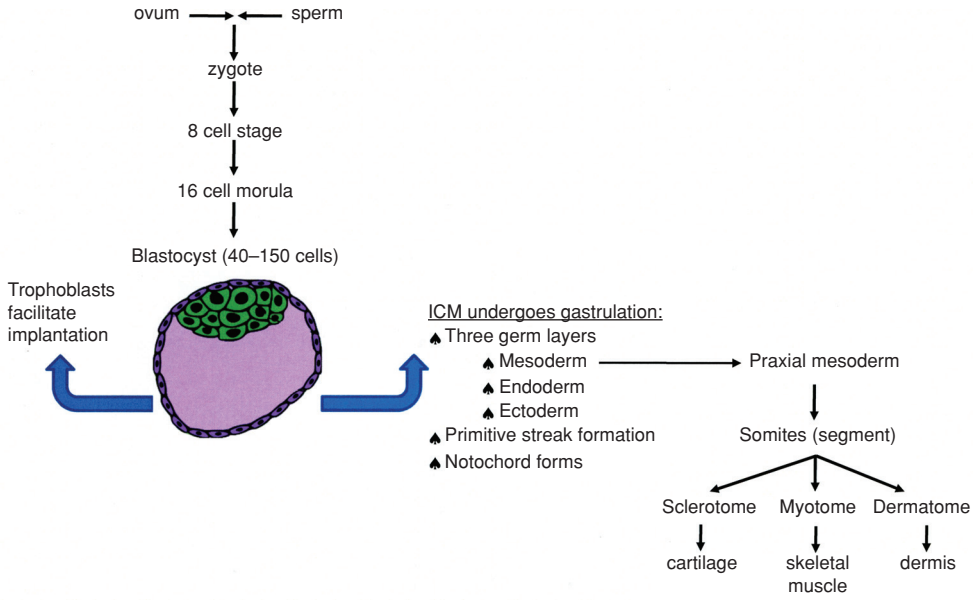

Figure 1.3 Development of the embryo and fates of cell populations found in the blastocyst. Cells of the inner cell mass give rise to the three germ layers subsequently forming the embryo proper. Trophoblast cells of the outer layer form the placenta.

Embryogenesis of Muscle

Skeletal muscle develops from a structural derivative of mesodermal somites of the early embryo identified as the myotome (Gilbert, 2006). Given that populations of muscle cell precursors arise during embryonic development, the discussion of muscle growth and development must begin with focus upon the development of the embryo.

Development begins at conception. During estrous, oocytes are released from the ovaries into the fallopian tube of the dam. Fertilization of the egg by the male sperm following copulation triggers activation of the oocyte and induces meiosis. The expulsion of polar bodies ensures the newly formed zygote contains a single set of male and female pronuclie (Fig. 1.3).

The events of early embryogenesis are very similar across mammalian species. Changes in gene expression dictate changes in cell function and fate. Initially, the two-cell zygote undergoes a period of rapid cell division characterized by an increase in cell number but a decrease in cell size. During this period, cells become tightly bound to one another causing the early embryo to assume a sphere-like shape. At this eight-cell stage, each cell of the embryo is totipotent, meaning they are completely unspecialized and capable of giving rise to any structure of the adult. However, beginning with the 16-cell morula, embryonic cells begin differentially expressing transcription factors via a poorly understood mechanism. Unique gene expression profiles drive cell specialization thus restricting the developmental fate of a given cell. For instance, cells in the outermost layer of the embryo at this stage begin to function as trophoblasts leading to the formation of a fluid-filled cavity that will become the blastocoel.

By the 40- to 150-cell stage, depending upon species, the developing embryo becomes a blastocyst that is capable of implanting into the endometrium of the uterine wall. At this point, the zona pellucida that surrounded the ovum has completely degraded and the embryo comprises two distinct

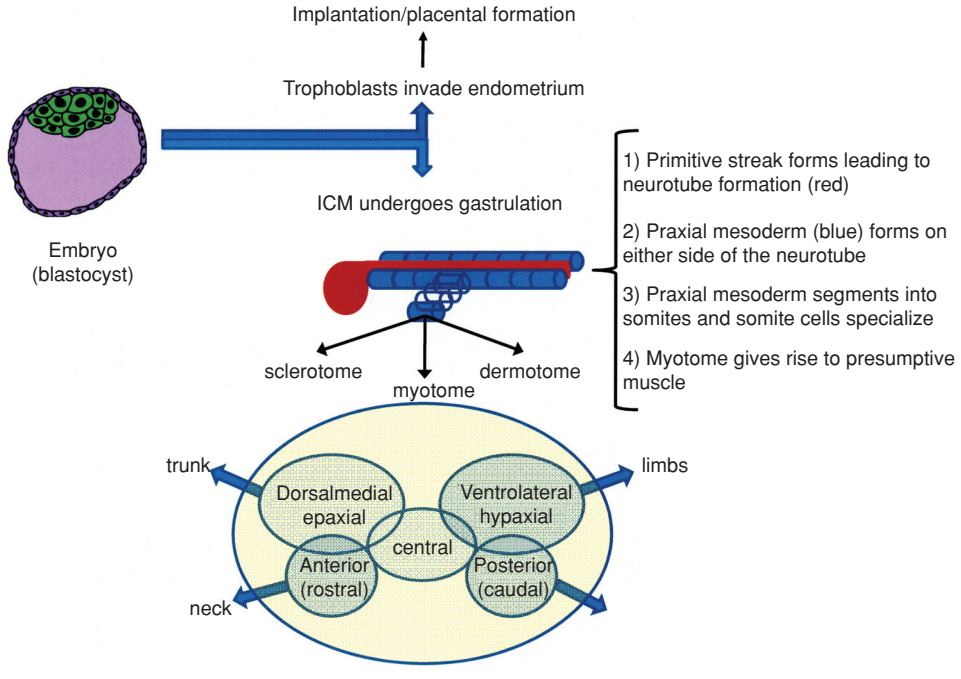

Figure 1.4 The derivation of the myotome and presumptive muscle.

cell populations, an outer layer of trophoblasts surrounding the well-formed blastocoel and a cluster of embryonic stem cells called the inner cell mass. The cells of the inner cell mass retain significant developmental potential whereas the cells of the trophoblast layer are more highly specified with their fate determined to cell types that largely support placentation (Gilbert, 2006).

At the time of implantation, two separate processes occur simultaneously within the blastocyst. First, trophoblasts differentiate into distinct layers. The outer syncytiotrophoblastic layer subsequently implants into the endometrium eventually developing into placental tissues. Cells of the second layer support placental growth. Meanwhile, as gastrulation is initiated, stem cells within the inner cell mass undergo a highly coordinated series of changes that eventually give rise to the three germ layers. Thus, trophoblasts facilitate implantation of the embryo and placental development while cells within the inner cell mass give rise to the mesoderm, endoderm, and ectoderm, three groups of cells from which all structures of the adult body will ultimately form.

Once cells of the mesoderm are specified, the stage is set for the development of presumptive muscle as the myotome is derived from the mesodermal layer (Fig. 1.4). Gastrulation is a period characterized by highly organized and coordinated cell migration and differentiation. During this period, the embryonic axis is set as the neural tube forms, the three pluripotent germ layers of the embryo become specified, and cells within embryonic germ layers become properly positioned. Following implantation of the embryo, the onset of gastrulation is signaled by the formation of the primitive streak. This structure emanates from the posterior end of the embryo as thickening of the epiblastic layer of the inner cell mass progresses. The paraxial mesoderm is formed as mesodermal cells migrate through the primitive streak and become positioned along either side of the developing neural tube. The paraxial mesoderm then segments into structures called somites. Cells of the somite further organize into three distinct masses via a poorly understood mechanism. Each cell mass gives

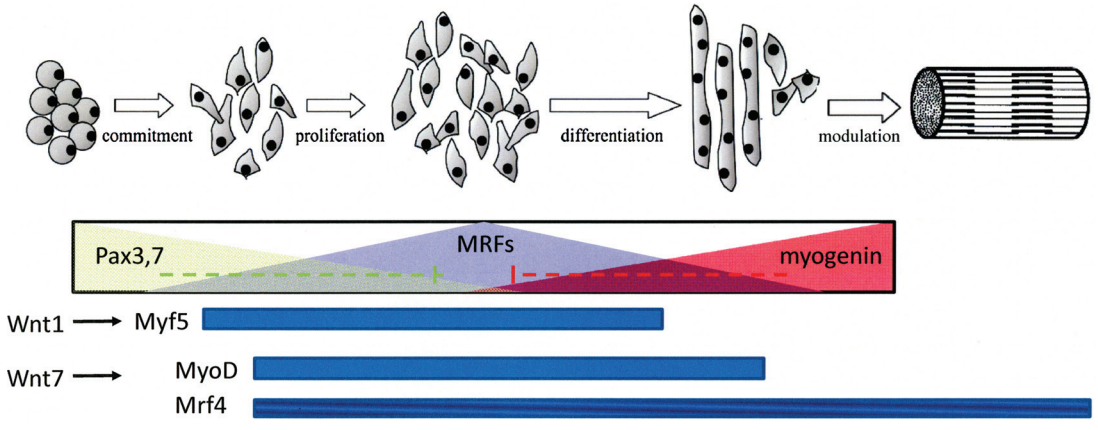

Figure 1.5 The basic events of myogenesis and the sequential changes in regulatory transcription factors that drive the process.

rise to distinct presumptive tissue. The sclerotome gives rise to cartilage. The dermatome gives rise to the dermis and the myotome gives rise to myoblasts (muscle precursor cells or muscle stem cells) that are capable of differentiating and fusing to become muscle fibers.

Myogenesis

Myogenesis is a complex multistage process. However, for the purposes of understanding, this process can be viewed as discreet phases encompassing muscle stem cell determination to myoblasts, proliferation of myoblasts, the differentiation and fusion of myoblasts to form myotubes, and the maturation of myotubes into mature muscle fibers (Fig. 1.5). Local signals from nonmyogenic cells direct the determination of somatic stem cells to the myogenic fate and orchestrate the highly ordered migration of myoblasts from the myotome through mesodermally derived extracellular matrix to sites of presumptive muscle (Sambasivan and Tajbakhsh, 2007; Buckingham and Montarras, 2008). Extracellular signals further direct the proliferation, fusion, and differentiation of myobasts into multinucleated primary myotubes. These multinucleated cells specialize as changes in the myogenic transcriptome give rise to proteins such as myosin, actin, tropomyosin, troponin, desmin, and *m*-isoform of creatine kinase that allow the newly formed myotube to function as a mature muscle fiber fully capable of contracting, metabolizing energy, and communicating with its surrounding environment (Buckingham and Montarras, 2008).

Sequential changes in gene expression drive the formation of skeletal muscle fibers from individual stem cells. Muscle stem cells are regulated by interactions between their intrinsic potential (as conferred by their genetic profile), their unique local environments, and distinct signaling pathways. The current paradigm for the regulation of muscle cell development indicates that myogenic signals converge upon three transcription factor networks to control entry into the myogenic lineage (Perry and Rudnick, 2000). These networks include the paired-box transcription factors (Pax), myogenic regulatory factors (MRFs), and myocyte-specific enhancer factors (MEFs). In this model, extracellular signals act on the stem cell to trigger signaling cascades which elicit changes in the expression of key transcription factors. These transcription factors, often acting as dimers, bind to *cis* elements in skeletal muscle-specific gene promoters allowing them to regulate the transcription rates of these

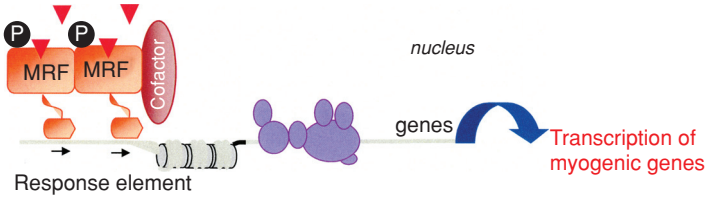

Figure 1.6 Muscle fiber formation occurs in a biphasic fashion from two separate populations of myoblasts during gestation. The precise timing of these events varies in a species-specific fashion.

genes (Fig. 1.6). Thus, altered transcription factor expression leads to changes in the expression of downstream genes resulting in altered cellular function.

Paired-box (Pax) genes are a family of transcription factors that regulate stem cell determination and tissue specification during early development of the embryo (Montarras et al., 2005). Nine family members have been identified to date based upon a conserved paired domain that dictates their DNA-binding specificity. Pax genes can further be grouped based upon their functional roles and tissue specificity. Importantly, expression of *Pax3* and *Pax7* (Pax group 3 genes) appears necessary for the emergence and survival of muscle stem cell precursors (Relaix et al., 2005; Relaix et al., 2006). Although it is now clear that Wnt signaling induces Pax3/7 expression, the extracellular factors that regulate cell fate through Pax3/7 are largely still unknown. Pax7 is essential for the developmental specification of muscle satellite cells playing roles in their maintenance and activation (Smith et al., 2001; Montarras et al., 2005).

Two additional families of transcription factors, the MRFs and the MEFs, play key roles in regulating myogenesis at steps downstream of the specification of stem cells to the myogenic lineage. MRFs are proteins that contain basic helix loop helix (bHLH) domains which facilitate specific DNA binding and protein–protein interactions that allow MRFs to transactivate muscle-specific gene promoters containing E-box motifs (Lassar et al., 1994; Ma et al., 1994). Key MRFs include myogenic determination factor 1 (MyoD), myogenic factor 5 (Myf5), myogenin, and myogenic factor 6 (Myf6/MRF4). MEFs such as MEF2 are MADS box transcription factors that potentiate myogenesis by synergizing with MRFs (Black et al., 1998; Black and Olson, 1998).

The roles that these transcription factors play in myogenesis have largely been delineated using cell culture models and murine knockout models. Through these experimental approaches, MyoD has been identified as a master regulator of myogenesis (Davis et al., 1987; Tapscott et al., 1988; Rudnicki et al., 1993). Among its myriad actions, MyoD functions to increase muscle precursor cell number both by driving the commitment of somite-derived Pax3/7 + precursors to the myoblast lineage and by stimulating the proliferation of existing myoblasts (Davis et al., 1987; Thayer et al., 1989). Muscle cells appear to arise from at least two lineages in the somite, though in both lineages paracrine factors restrict development to the muscle fate by inducing MyoD expression. In hypaxial muscle, Pax3 activates MyoD expression in the absence of inhibitory transcription factors. However, in epaxial muscle, expression of Myf5 induces MyoD expression. Regardless of the events leading to MyoD expression, MyoD appears to be a primary trigger signaling myogenic commitment. Although Myf5 has considerable functional redundancy with MyoD and Myf5 similarly stimulates myoblast proliferation, its precise role as a myogenic regulator remains controversial.

Other MRFs regulate myogenesis downstream of myogenic commitment. Myogenin regulates myoblast differentiation and fusion (Edmondson and Olson, 1989; Wright et al., 1989). Curiously, MRF4 has also been implicated in the differentiation and fusion of myoblasts during the formation of primary embryonic fibers but not during secondary fetal fiber myogenesis (Rhodes and Konieczny,

1989). MRF4 also plays a role in the maintenance of the myofiber transcriptome in adult muscle serving to induce many genes important for myofiber structure and function.

The regulation of myogenic determination is poorly understood and the literature addressing this issue is currently controversial. However, the regulation of myoblast differentiation is better characterized. Through studies examining myogenic gene expression during myogenesis, a regulatory cascade of MRF gene expression that is essential for myogenesis has emerged. As a result, Pax genes and MRFs also serve as muscle cell markers given their temporal pattern of expression plays a vital role in myogenesis. In this model, muscle stem cell progenitors express Pax3/7. Signals triggered by extracellular factors such as fibroblast growth factor (FGF) drive the determination of myoblasts by down-regulating the expression of Pax group 3 genes concomitant with the induction of MyoD and Myf5. Proliferating myoblasts express MyoD and Myf5 with increased myogenin expression only occurring immediately before myoblast differentiation. Finally, MRF4 expression is upregulated upon terminal differentiation of the myoblast. Myogenesis can be manipulated both *in vitro* and *in vivo* through altering these expression patterns either through knocking out or overexpressing individual MRFs. Thus, myogenesis is directed by the temporally controlled expression of MRFs which sequentially stimulate or repress muscle-specific gene promoters. This allows finite control of structural, contractile, and enzymatic protein expression that is essential for muscle cell function.

Somite-derived myoblasts are the only cell populations identified to date that can contribute to increased muscle fiber number. Importantly, myoblasts represent a pool of muscle cell precursors that can further expand in response to mitogenic signals. Myoblasts can either progress through the cell cycle when exposed to growth factors or they can remain replication-competent yet dormant. However, despite being committed to the muscle cell lineage, myoblasts remain unspecialized, meaning they do not yet express genes that are integral to conferring muscle function. Thus, while increased myoblast numbers favor greater muscle fiber formation, an increased number of myoblasts does not lead to increased fiber number *per se*.

In order to gain muscle function, myoblasts must first exit the cell cycle and differentiate before subsequently fusing to form myotubes. Importantly, as illustrated in Figure 1.5, these processes are distinct and controlled by different regulatory mechanisms. In the absence of mitogenic cues such as FGF, myoblasts can be directed to exit the cell cycle. Such myoblasts become intertwined in a network of connective tissue facilitated in part through their ability to secrete fibronectin to which integrin receptors anchor. This interaction between integrins and fibronectin appears necessary for myoblasts to differentiate (Menko and Boettiger, 1987; Rosen et al., 1992). Extracellular cues such as growth factors, hormones, cytokines, and components of the extracellular matrix drive the changes in gene expression that underlie these developmental changes (Hollis, 1993; Doumit et al., 1996). Thus, prenatal events that govern the extent of myoblast proliferation and the timing of differentiation ultimately determine the number of fibers a muscle will contain in postnatal life.

The fusion of myoblasts into multinucleated cells likewise depends upon extracellular factors. Differentiation-competent myoblasts align through cell surface-specific interactions mediated by cadherins and cell adhesion molecules expressed on the myoblast cell membrane (Mege et al., 1992). Once aligned, calcium ionophores allow an influx of calcium into the myoblast cytosol thus activating a set of metalloproteinases called meltrins which mediate membrane fusion in a poorly understood process. Concomitantly, myogenin is expressed leading to terminal differentiation of the myoblast through activation of a host of muscle-specific genes. The differentiated, fused myoblasts now comprise a multinucleated, immature muscle fiber termed a myotube. Further recruitment and fusion of myoblasts coupled with the expression of muscle proteins such as creatine kinase, myosin, and actin results in a mature, contraction-competent muscle fiber. Given that the primary function of muscle is contraction, myofibrillogenesis is an integral part of muscle fiber maturation. The regulation of myofibril development is poorly characterized at this time, however.

Biphasic Fiber Formation

Myoblasts are derived from the myotome and migrate from somites to sites of presumptive muscle where they proliferate, aggregate, align, and fuse into myotubes. Muscle fiber number increases in a biphasic fashion with an embryonic wave giving rise to primary fibers and a second fetal wave giving rise to secondary fibers. The timing of these waves of myogenesis occurs at species-specific gestational ages though ultimately total muscle fiber number is set by birth in most species.

Primary fibers first form from a population of embryonic myoblasts. The regulation of this process is poorly understood but it is thought that primary fiber number dictates the ultimate potential fiber number of the adult animal (Du et al., 2009). This is because these initial fibers serve as a scaffold that is critical for the alignment and fusion of secondary myoblasts. Thus, the number and cross-sectional area of primary fibers greatly impacts the potential for secondary fiber formation. Eventually, the innervation of primary fibers and their subsequent contraction breaks associations between primary and secondary fibers allowing individual muscle cells to become encapsulated by their own basal lamina (Fredette and Landmesser, 1991; Wilson and Harris, 1993). Although species-specific differences exist in the timing of these events and the number of secondary fibers that associates with each primary fiber, the biphasic nature of fiber development is conserved across species (Gerrard and Grant, 2003). In general, muscle fiber number is set by the time the fetus reaches the final third stage of gestation. Finally, not all myoblasts fuse to form new fibers, becoming instead satellite cells that are destined to fuse only with existing fibers in mature whole muscle (Fig. 1.7).

Myocytes, adipocytes, and fibroblasts all are derived from the mesoderm (Gilbert, 2006). Although the timing is species-specific, the formation of secondary fibers coincides with the onset of adipogenesis and fibrogenesis during mid- to late gestation. Thus, secondary fibers form during a time when populations of fibroblasts are beginning to secrete extracellular matrix components and presumptive adipose tissue is appearing. Given it is now known that adipocytes, muscle fibers, and fibroblasts secrete growth factors and cytokines, it seems likely that crosstalk between these emerging cell types may serve to coordinate the development of whole muscle by influencing fiber number, the development of the perimysium and epimysium layers, and influencing the potential to deposit intramuscular fat.

Given that increasing muscle mass is desirable from a profit standpoint, strategies which increased primary fiber number or size would be desirable. Unfortunately, primary fiber number has proven to be intractable to manipulations of the intrauterine environment (Russell and Oteruelo, 1981; Du et al., 2009). However, primary fiber number is significantly influenced by breed suggesting that it may be possible to select for increased primary fiber number (Stickland and Handel, 1986). Encouragingly, muscle mass can be manipulated within breed and several studies have now shown that primary fiber size can indeed be altered by manipulating the gestational environment as factors such as nutritional plane and exposure to factors such as IGF-1 can influence primary fiber hypertrophy (Du et al., 2009).

Hypertrophy (Postnatal Muscle Development)

As fiber number is set at birth in mammals, postnatal increases in muscle mass primarily occur through the hypertrophy of existing fibers. This growth is accomplished by increases in both the length and diameter of muscle fibers. Fiber size can also be modulated through changes in the fiber

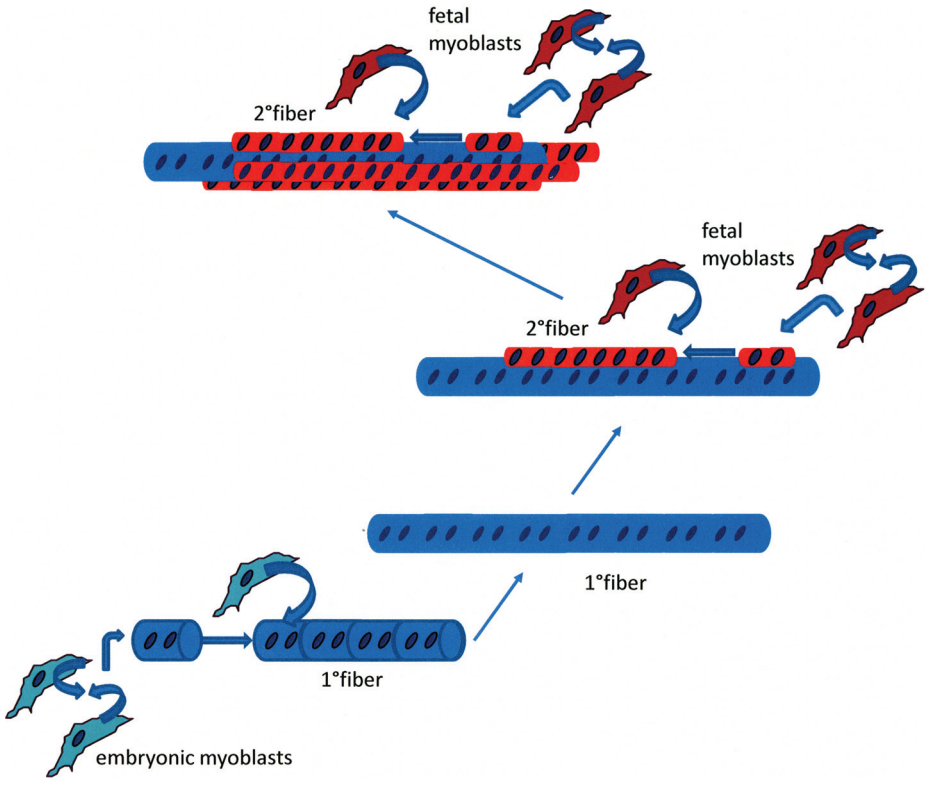

Figure 1.7 Mechanism for the transactivation of muscle-specific genes by MRFs. MRFs bind to specific response elements in the regulatory region of genes where they recruit other proteins forming a complex that promotes assembly of the transcriptional machinery leading to initiation of transcription. Signaling events can modulate this process through reversible phosphorylation of MRFs.

type composition of an individual muscle as, for instance, a switch from smaller oxidative fibers to the larger glycolytic fibers would be associated with an increase in muscle mass.

Regardless of the mechanism, increases in muscle mass are supported by dramatic reorganization of the contractile apparatus. Dynamic in nature, myofibrils can increase in size by the addition of sarcomeres at either ends of the myofibril and through the addition of thick and thin filaments to the periphery of the myofiber. Upon reaching a critical thickness, growing myofibrils can also split into new myofibrils via a poorly understood mechanism.

The characteristically large size and specialized contractile function of the muscle fiber imposes unique constraints upon its hypertrophy. Myofibrillar proteins comprise approximately 60% of total skeletal muscle protein (and may significantly impact meat texture). Consequently, the synthesis of myofibril regulatory proteins, the myofibril itself, and the maintenance of its integrity represents a tremendous burden upon the nuclei of the muscle cell. Thus, increases in muscle fiber size are only possible when rates of protein synthesis are significantly greater than rates of protein degradation. Therefore, the rate of postnatal muscle growth is predominantly a function of the balance between protein synthesis and degradation except for minor postnatal changes in fiber number associated with injury-stimulated fiber regeneration. Also, as the volume of sarcoplasm that can be supported by a single nucleus is limited by the rate of diffusion, increases in muscle fiber size are largely limited

by the ability of available nuclei to support myofibril protein synthesis. During postnatal growth, the number of nuclei in muscle cells increases substantially as evidenced by a 2- to 100-fold increase in the DNA content of the muscle cell (Gerrard and Grant, 2003). As the terminally differentiated myocyte is incapable of reentering the cell cycle, the potential for postnatal muscle fiber hypertrophy is dictated by the degree that satellite cells residing within the basal lamina contribute their nuclei through fusion with the growing muscle fiber (Montarras et al., 2005).

Satellite Cells

Satellite cells are a population of adult, mononucleated myoblasts that are sandwiched between the basal lamina and the sarcolemma of mature muscle fibers. Their primary functions are to act as a pool of nuclei that supports muscle fiber hypertrophy and facilitates muscle fiber regeneration following injury. There appear to be two populations of satellite cells in adult muscle (Montarras et al., 2005). One population resides within the basal lamina in close proximity to mature muscle fibers thus allowing their fusion. Even though satellite cells are replication competent, the presence of the basal lamina likely prevents them from forming new fibers by representing a physical barrier that prevents their aggregation and alignment. A second population of satellite cells is best characterized as a Wnt-activated muscle stem cell that differentiates in response to an injury-stimulated cascade (Montarras et al., 2005). Trauma to the fiber induces Wnt signaling in such cells resulting in the induction of Pax7 and ultimately leading to activation of MyoD family of genes. It is possible that subpopulations within these groups may underlie divergence in fiber type in the mature animal.

Generally, satellite cell number decreases with increasing muscle mass and animal age though satellite cell proliferation occurs throughout growth ceasing only when lipid deposition becomes appreciable in muscle (Trinkle et al., 1978). Satellite cells exist either as quiescent (reside in the G0 stage of the cell cycle) or active cells, and their behavior in culture mimics that of embryonic myoblasts with regard to an ability to proliferate, differentiate, and respond to mitogens and other known regulatory signals (Florini and Magri, 1989; Allen and Rankin, 1990).

It is currently unclear how satellite cell fusion is directed by the enlarging muscle fiber though it is clear that muscle fibers secrete cytokines and growth factors that are known to affect satellite cell activation, proliferation, and differentiation (Pedersen and Febbraio, 2008). *In vitro* experiments indicate that a myriad of growth factors regulate all phases of the satellite cell life cycle (Allen and Rankin, 1990). Importantly, since heparin sulfate and other proteoglycans abundantly expressed in the extracellular matrix associate with growth factors, the basil lamina represents a rich site of sequestered growth factors. Presumably, such growth factors can interact with satellite cells within the basal lamina and readily influence the satellite cell life cycle. Certainly, the location of satellite cells in close proximity with the sarcolemma suggests crosstalk between muscle and satellite cells is likely.

Protein Turnover

Proteins are constantly turned over in skeletal muscle by undergoing a continuous cycle of degradation and resynthesis. Although this cycling may seem wasteful and indeed represents a drag upon protein accretion rates in the growing animal, protein turnover allows muscle cells to rapidly regulate protein concentrations and remove improperly folded or damaged proteins. As a result, muscle cells are better able to adapt to changing external requirements.

Protein Synthesis

Significant amounts of protein synthesis are required to support increases in muscle fiber length and diameter. The central dogma describes the flow of biological information in cells leading to the expression of functional proteins. In this paradigm, messenger RNA (mRNA) is transcribed from genes specified by the nucleotide sequence of DNA. We have already seen how myogenic transcription factors direct the transcription of muscle-specific genes to mRNA during myogenesis through their interaction with the regulatory regions of these genes. Once transcribed and processed, mRNA is translocated from the nucleus to the cytosol where it interacts with ribosomes. The nucleotide sequence contained in the mRNA serves as a template specifying the specific amino acids that should be added to the growing peptide chain by interactions with small aminoacyl-tRNA. Finally, the newly synthesized peptide chain gets modified post-translationally.

Importantly, there are physical limits to the amount of cytosol a nucleus can efficiently supply with protein. The range is essentially set by the rate in which the nucleus can generate the mRNA species required to support the protein needs of the cell in light of the turnover rate of the given protein (Allen et al., 1999). As seen earlier, this problem is solved by the muscle cell being multinucleated with satellite cells serving as a pool of nuclei that can support increasing size of the muscle cell.

Protein translation, the conversion of the mRNA sequence to a growing peptide chain on the ribosome, is also a highly regulated process that occurs in three sequential stages (Pain, 1986). First, an initiation complex is formed through interaction of ribosomal subunits, the mRNA species being translated, and an initiator aminoacyl-tRNA. Second, the peptide is elongated as amino acids are added to the growing chain. Finally, the chain is terminated in response to a stop codon on the mRNA and the polypeptide chain gets processed and folded into the functional protein. Each step of translation represents a point of regulatory control and could be a rate-limiting step in the process of protein synthesis.

Protein Degradation

Protein degradation is the breakdown of proteins into smaller polypeptides and individual amino acids by proteolytic enzymes. If not counterbalanced by new synthesis, protein degradative pathways will decrease the amount of functional protein expressed by the cell. Given that proteins continually turnover or go through a reoccurring process of protein degradation and resynthesis, there is a constant need for new protein synthesis in order to maintain myofibril integrity and fiber size.

Traditionally, emphasis was placed on better understanding the regulation of protein synthesis, so this is a well-understood process. However, there are generally upper limits to which protein synthesis can occur in growing muscle. Therefore, much recent effort has been devoted to better understanding the regulation of protein degradation in the hopes of increasing protein accretion by limiting protein degradation rates.

Generally, protein turnover rates are much greater than protein accretion rates in a given muscle. Half lives for muscle proteins can range from 2 to 20 days depending upon the protein. Turnover rate is generally a function of the class of proteins being most rapid for sarcoplasmic proteins, intermediate for myofibrillar proteins, and slowest for stromal proteins (Gerrard and Grant, 2003).

There are currently three well-characterized proteolytic systems known in skeletal muscle. Lysosomal proteolysis is carried out in organelles that form a specialized cellular compartment equipped with proteases such as cathepsins that function optimally at low pH. Lysosomal proteolysis accounts for approximately 20–30% of the protein turnover in muscle fibers and specifically targets sarcoplasmic proteins (Lowell et al., 1986a, 1986b). The lower pH of the cytosol, presence of cytoplasmic cathepsin inhibitors such as cystatins, and the lack of evidence for myofibril transport into lysosomes suggests lysosomal proteolysis plays little role in the turnover of myofibrillar proteins.

A second pathway, the ubiquitin-proteosome proteolytic pathway, targets regulatory proteins, short-lived proteins, and sarcomeric proteins (Mitch and Goldberg, 1996). Multiple enzymes in this system function to attach ubiquitin to proteins that have become destined for degradation. Polyubiquinated proteins are subsequently trafficked to 26S proteosomal complexes where they are broken down. Inhibiting the proteosome-ubiquitin pathway significantly lowers proteolytic breakdown suggesting that this pathway plays a significant role in protein degradation in muscle cells (Rock et al., 1994; Tawa et al., 1997).

Finally, a third system of proteases was discovered, the calpain system, due to the ability of these proteases to act directly on Z-discs from myofibrils (Bullard et al., 1990). Calpains are activated by intracellular calcium levels and were initially grouped based upon their calcium sensitivity *in vitro*. For instance, μ-calpain can be activated at micromolar concentrations of Ca while m-calpain requires millimolar amounts. The μ- and m-calpains are termed ubiquitous calpains because they are expressed in all tissues. A third calpain, p94 or skeletal muscle calpain, is specifically expressed in muscle. The activities of the ubiquitous calpains increase in conditions associated with muscle degradation. Although considerably less is known about muscle calpain, it seems to serve an opposite role by protecting the muscle from proteolysis (Gailly et al., 2007; Lamb, 2007). Calpastatin inhibits the activity of the ubiquitous calpains, it too being regulated by intracellular calcium levels. Finally, phosphorylation of calpains can modulate their requirement for calcium thus representing a further layer of regulation directing this important proteolytic system. Since calpains are integral in directing postmortem proteolysis, the calpain system is especially relevant to meat quality because of their influence on meat tenderness.

Muscle Cell Culture

During the past 20 years, cell culture techniques have taken on great importance for the study of muscle biology. Cell culture has facilitated the study of the transcriptional regulation of myogenesis, the hormonal regulation of protein turnover, and the control of nutrient uptake by muscle in ways that would otherwise be impossible *in vivo*. Importantly, cell culture systems facilitate mechanistic inquiry that can be useful for *in vivo* studies. Most recently, it has been revealed that muscle cells release cytokines and growth factors to a much greater extent than previously appreciated. Thus, myofibers may also participate in a peripheral endocrine axis between adipose tissue and skeletal muscle that modulates the effect of the hypothalamic–pituitary–gonadal axis on growth. Surely, skeletal muscle culture systems will be instrumental in further elucidating this emerging endocrine function of muscle fibers.

Essentially, all known mitogens and endocrine factors effecting myogenesis have been discovered through *in vitro* experiments. Gene expression studies using cell lines and primary cultures have revealed expression patterns that suggest regulatory roles. In many cases, these observations have been extended using gene overexpression and knockdown experiments. It was in this way that the original roles for MRFs were demonstrated using murine C3H $10T\frac{1}{2}$ nonmuscle embryonic stem cells. Azacytidine is an analog of cytidine that incorporates into DNA and blocks methylation thus preventing gene silencing. It was observed that treating $10T\frac{1}{2}$ cells with azacytidine could induce them to specify cartilage, fat, or muscle. Using this model, MyoD was subsequently discovered by a subtractive hybridization approach and it was demonstrated that the transfection of MyoD alone was sufficient to convert fibroblasts to myoblasts (Davis et al., 1987; Tapscott et al., 1988). These observations were followed up by similar overexpression studies which established myogenin, myf5, and MRF4 as important myogenic regulators and helped to elucidate the MRF transcriptional cascade discussed in Figure 1.5 (Edmondson and Olson, 1989; Rhodes and Konieczny, 1989; Wright

et al., 1989). The MRF family is unique in its ability to drive the transdifferentiation of nonmuscle cells to myoblasts. These data suggested its members are the master regulators of myogenesis and formed the basis for conducting more elaborate and costly experiments using transgenic animal models. As a result, there has been an explosion in our understanding of the regulation of myogenesis in the past decade.

A good deal of this chapter has been devoted to discussing the regulation of muscle cell hyperplasia and hypertrophy. We will next focus on the cell culture techniques that have allowed this regulation to be studied in great detail.

Basic Concepts of Cell Culture

Tissue or cell culture basically involves harvesting tissue or cells from the live animal and incubating them in a plastic dish. Thus, cell culture is synonymous with the term *in vitro* as it is the *in vitro* process of growing cells artificially in the laboratory. The definition of *in vitro* and *in vivo* research depends on the experimental model used. *In vivo* literally means within a living organism and refers to experimentation conducted in the intact organism. *In vitro* literally means "in glass" but generally refers to any artificial environment that is outside the body. For the purposes of cell culture, *in vivo* refers to experiments conducted in living animals and *in vitro* refers to experiments that use living cells propagated in an artificial environment outside of the animal. The purpose of this section is to provide a conversational knowledge of how researchers conduct cell culture.

The ultimate goal of cell culture is to mimic the *in vivo* situation by creating an *in vitro* environment such that the cells of interest model or maintain the behavior they would exhibit in the animal as closely as possible. This approach affords researchers significant advantages but they are not without important caveats.

Given the goal of cell culture is to create an environment that mimics the *in vivo* state, there are several critical requirements for successfully culturing cells *in vitro*. First, cells must be incubated in an environment that maintains the appropriate temperature and pH which in most cases is 37°C and a pH of 7.2. Cells must also be supplied a suitable growth medium that contains energy substrates, amino acids, and inorganic salts in sufficient quantities to support cell metabolism and growth. This medium can be provided as a liquid or semisolid depending upon the requirements of cell type being utilized. Growth regulators must also be provided as cells will not progress through the cell cycle without exposure to mitogens and growth factors. The specific requirements for sustained proliferation are often not well characterized for many cell types, so this criterion is most often satisfied by supplementing the base medium with serum from either bovine fetal or calf sources. One disadvantage of serum supplementation, however, is that serum is an undefined component that can display dramatic variation from one lot to the next so there is the potential that serum supplementation can confound experimental results. In many instances, serum-free conditions can be worked out to facilitate experiments that do not require cells to proliferate. Furthermore, several companies now make defined cocktails of mitogens and growth factors that may allow certain cultures to proliferate in serum-free conditions. Finally, it is vital that cell culture be conducted in aseptic (sterile) conditions. Microorganisms are virtually impossible to eradicate once in culture and they grow much more quickly than mammalian cells allowing them to quickly outcompete cells of interest for available resources. Ultimately, the need to meet these critical parameters will dictate how cell culture is performed in the laboratory.

There are two major classes of cell culture models—primary culture and continuous culture—that are defined by how the cells are derived and by their growth characteristics. Each system offers unique advantages and disadvantages. These are important to consider when determining the optimal

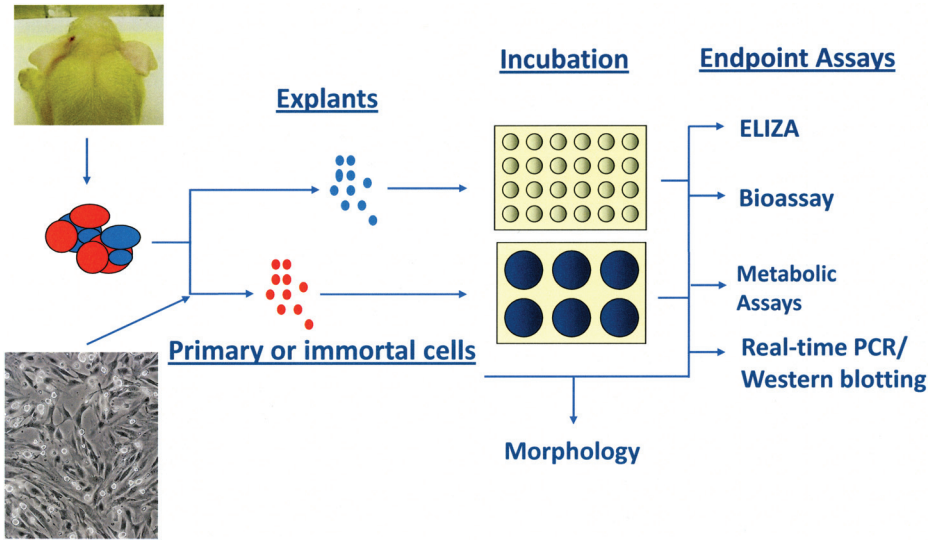

Figure 1.8 Generalized schematic for the use of cell culture in animal experimentation.

system to adopt and the degree to which data generated by such systems can be extrapolated to other models (Fig. 1.8).

The first model system is termed primary culture because cells are derived directly from excised, normal animal tissue. In this system, the culture can be incubated as either explants or as single-cell suspensions. Explants are essentially small chunks of tissue that maintain the cytoarchitecture of the original tissue. This may be advantageous depending upon the question being asked by the investigator. In contrast to explants, single-cell suspensions are derived by the dissociation of harvested tissue through the enzymatic digestion of connective tissue and extracellular matrix components (most often via collagenase). Cell suspensions allow the culture of specific cell types as opposed to the highly heterogeneous nature of explants. This allows the researcher to avoid confounding effects caused by the potential crosstalk between different cell types in explants. A major advantage of primary culture is that the cells usually retain the characteristics they display *in vivo* to a large degree once cultured *in vitro*. Thus, primary culture represents the most faithful system to model behavior in the animal. However, primary cultures can only be maintained for a limited time as primary cells senesce or exit the cell cycle after a limited number of cell divisions. Explants tend to undergo necrosis if chronically incubated. Furthermore, primary cultures often represent a heterogeneous population of cells that is largely undefined making mechanistic inquiries more difficult. Also, primary cultures will retain the genetic variation inherent in the animal from which they were harvested. Thus, primary culture necessitates the sacrifice of animals which are undesirable both from a cost standpoint and from the need to minimize animal suffering when conducting animal research.

The second major class of cell culture models is termed continuous culture because these cultures comprise immortalized cells. These cells have been cloned originally from an animal source but now are considered to exist as a homogeneous population that can be propagated indefinitely. Thus, continuous cultures are described as being cell lines. Cell lines are often derived from tumors, hence their immortal character. Alternatively, they can be stem cells or cells that have been immortalized chemically or via transfection of foreign DNA that disrupts the normal regulation of their cell cycle.

Cell lines offer several advantages in that they provide an endless supply of cells that should not vary greatly in their phenotype. Cell lines are often very easy to transfect facilitating mechanistic inquiry and overexpression/knockout studies. However, cell lines often have chromosomal abnormalities that arise from their continuous propagation. Thus, even with cell lines, phenotypic drift is a major concern. Furthermore, since they are often derived from tumors, the ability to extrapolate data back to the live animal may be limited.

Given the caveats associated with primary and continuous cultures, one might ask, why do cell culture at all? Despite the concerns associated with conducting *in vitro* experiments, cell culture offers many important advantages. First, cell culture systems allow growth biologists to conduct experiments that would otherwise be impossible *in vivo*. Furthermore, *in vitro* experimentation allows the investigator to control variation to a much greater degree thus making it more likely that the questions being asked will be answered more reliably. For instance, the investigator can control the physical and chemical environment, alter physiological conditions at will, as well as finely manipulate critical parameters such as concentration and time, all of which may be essential variables of an experiment. Importantly, cell culture can serve to limit the use of animals and the homogeneity of cell cultures can aid in interpreting experimental results as well as improving reproducibility.

It is paramount to practice proper aseptic technique in order conduct successful cell culture experiments. While providing cells with a complete medium, a warm and humid environment, and a suitable pH is essential to maintain both their growth and a desirable phenotype; doing so also creates an environment that promotes the growth of undesirable organisms such as microbes, yeasts, and molds.

A primary tenant of sterile technique is that all work should be conducted in a sterile environment. This requires the use of a cell culture hood (alternatively called a biological safety cabinet) and chemical disinfectants the most common being a 70% alcohol solution. A class II laminar flow cabinet maintains a sterile environment by circulating air through a system of hepa filters such that the working environment is bathed in sterile air. It is important to appreciate the direction of airflow so that vents supplying the filtration system are not blocked. Furthermore, this knowledge allows motion to be minimized between the filter and the cultures. This is important as the primary source of contamination is in fact the user. Finally, the sash on the front of the hood serves to both minimize undesirable air currents created by movement outside of the hood as well as to protect the user from aerosols that are an unavoidable part of manipulating cultures and growth medium. Liberally spraying down work surfaces with a disinfectant can greatly mitigate the potential hazard posed by aerosols as well as prevent the growth of undesirable contaminants.

Density dependence is another important characteristic that must be considered as it dictates how cultures are handled. Cells can be grouped by morphology based upon whether they adhere to a surface and thus grow as a monolayer (as do most cells derived from tissue) or whether they float in culture and are thus grown in suspension (as do cells derived from the blood). Muscle cell cultures are adherent cultures thus their density can be described based upon the percent confluence of their monolayer. Percent confluence is determined roughly by the degree to which cells have achieved a state where they are in maximal contact with neighboring cells. Whether a particular culture has rigorous density dependence dictates the percent confluence the cultures can be allowed to reach. For instance, some cultures are not inhibited by cell-to-cell contact and thus will continue to divide regardless of whether confluence of the monolayer is achieved. Other cells will exit the cell cycle when cell-to-cell contact is achieved via a process called contact inhibition. Thus, allowing such cells to reach confluence could have dramatic consequences for the character of the cell culture being propagated.

As a result of their density dependence, the continuous propagation of adherent cell cultures generally requires that they be subcultured or "passaged". Passaging a culture simply involves disruption of the monolayer by protease treatment to create a cell suspension that can then be easily diluted. Fittingly, this process is termed a passage. In this way, cell density can be managed and cells can be obtained and reseeded for use in experiments. The most common protease used for passaging cultures is trypsin. Passage number is an important parameter to monitor as the character of primary cultures or a continuous cell line will inherently drift with increasing passage number. Thus, the behavior of cells can be altered greatly with time especially if cultures are not handled in a consistent manner.

The characteristic drift seen with the continuous propagation of cells in culture can be managed by the cryopreservation of large numbers of cell stocks that have been harvested at a low passage number. Cryopreservation involves the freezing and subsequent storage of cell stocks at ultralow temperatures. Storage is generally accomplished by maintaining cell stocks in the liquid nitrogen vapor phase within a cryogenic tank. Generally, cultures are frozen at an optimal cooling rate in the presence of serum and a cryoprotectant such as dimethyl sulfoxide (DMSO) or bovine serum albumin (BSA) that serves to prevent crystal formation and damage to the cell due to dehydration. By limiting the number of times a culture can be passaged before being terminated in favor of revitalizing a frozen stock, the character and phenotype of a continuous cell line can be maintained within narrow parameters thus allowing the maximal degree of repeatability.

Culture of Established Muscle Cell Lines—Murine C2C12 Myoblasts

The C2 myoblast cell line was originally isolated from C3H mice by causing trauma to their leg muscles in order to stimulate satellite cell proliferation (Yaffe and Saxel, 1977). The resultant cloned myoblasts maintain fibroblastic character in the presence of high levels of fetal calf serum. However, they can be induced to differentiate and fuse by withdrawing serum. Importantly, C2 myoblasts express MyoD, Myogenin, and Myf5 (Neville et al., 1997) making them an excellent model for studying myogenesis. The C2C12 mouse myoblast cell line is a diploid subclone of the C2 parent line selected on the basis of its ability to fuse rapidly to form contracting myotubes which highly express muscle-specific proteins (Blau et al., 1985a, 1985b). The C2C12 fusion is enhanced in confluent cultures by withdrawing fetal bovine serum from media and supplementing it instead with lower amounts of horse serum. Recently, it has been demonstrated that the overexpression of bone morphogenic protein 2 (BMP-2) induces C2C12 cultures to differentiate into osteoblasts suggesting the specialization of this cell line is not fixed solely to the myogenic lineage (Partridge et al., 2002).

Due to their growth properties, C2C12 myoblast cultures require constant maintenance. Myoblasts are spontaneously induced to differentiate by cell-to-cell contact. Since C2C12 myoblasts have a short doubling time (16 h) and tend to grow in clumps resembling the formation of colonies, it is necessary to monitor C2C12 cultures daily and to passage them often in order to avoid counter-productive selection pressures. Generally, cultures should not be allowed to grow to greater than 50–70% confluence. Allowing cell density to become too high selects for slower fusing myoblasts because fast-fusing ones will exit the cell cycle and drop out of culture as they differentiate. Similarly, due to their fast-fusing nature, cultures of C2C12 myoblasts are more susceptible to drift in their character with increasing passage number than most fibroblastic cell lines. This necessitates that large quantities of C2C12 myoblasts be cryopreserved at a low passage number so that fresh vials of cells closely resembling the character of the original culture can be thawed and seeded in order

to mitigate issues related to their continuous culture. Nonetheless, their rapid growth, fast-fusing character, ability to be easily transfected, and their robust expression of MRFs and muscle-specific genes makes the C2C12 myoblast cell line a valuable tool for studying myogenesis and muscle biology.

The procedures that follow have been adapted from the recommendations of American Tissue Type Collections for C2C12 myoblasts (ATCC® catalog number: CRL-1772) through the routine use of this cell line in the Brandebourg Laboratory. These protocols are meant to serve as a practical guide for the use of this cell line in teaching and research endeavors.

Propagation

Cultures are propagated at 37°C in a 5% CO_2 environment. Growth medium consists of Dulbecco's Modified Eagle's Medium (DMEM) formulated to contain 4.5 g/L glucose, 1 mM sodium pyruvate, 4 mM L-glutamine, 15 mM Na_2CO_3 (optimal for 5% CO_2 environment), a phenol pH indicator, and supplemented with fetal bovine serum to a final concentration of 10%.

Cultures should be observed everyday and evaluated for percent confluence, doubling rate, uneven distribution, and for signs of fusion. Growth medium should be changed every 2 days. Generally, C2C12 cultures seeded in a 75 cm^2 culture flask require 10 mL of medium per 48 h period in culture. This suggested volume to surface area is scalable to different-sized flasks. However, no more than 15 mL of medium should be added to a 75 cm^2 culture flask as too large a volume of medium can adversely affect cultures due to weight/pressure issues. Cultures should be subcultured (passaged) before the monolayer reaches no greater than 50–70% confluence.

Subculturing

The passage of cells that grow as a monolayer in culture requires the disruption of their attachments to one another and to the surface of the culture vessel in order to create a cell suspension that makes pipetting and diluting possible. It is important that cultures not be allowed to reach confluence. Multiple 75 cm^2 flasks can be used in order to achieve a sufficient yield of cells despite the strict passage of cultures when the monolayer reaches 50% confluence.

(1) Aspirate and discard the conditioned growth medium using a mild vacuum.
(2) Since the growth medium contains serum, rinse the culture flask with several milliliters of a balanced salt solution (BSS) that is congruent with the BSS used to make the trypsin solution (normally calcium-free Hanks).
(3) Add 2 mL of a 0.25% (w/v) Trypsin-0.53 mM EDTA solution per 75 cm^2 of surface area in the culture flask and incubate the flask at 37°C for 3–5 minutes or until the monolayer is completely dissociated. Check to ensure that all cells have come off of the plate by examining the cell suspension under an inverted microscope. It may be necessary to gently tap the culture flask to encourage cells to dissociate from the flask surface but do not use excessive swirling as this promotes clumping of cells.
(4) Once digestion is complete, add an equal volume of serum-containing growth medium to the culture flask and gently agitate the cell suspension with pipette action to allow trypsin inhibitors in the serum to inactivate the trypsin solution.
(5) Sample an aliquot of the cell suspension in order to perform cell counting. Seed culture plates for experiments or new culture flasks by adding an appropriate volume of the cell suspension to the new plastic vessel in the presence of a sufficient volume of growth medium. Serum aids in cell attachment, so seeding medium will require the presence of serum regardless of whether subsequent experiments will be conducted under serum-free conditions. Avoid leaving a cell suspension on fresh plastic for any period of time without addition of growth medium as doing so promotes an uneven distribution of attached cells (Fig. 1.9).

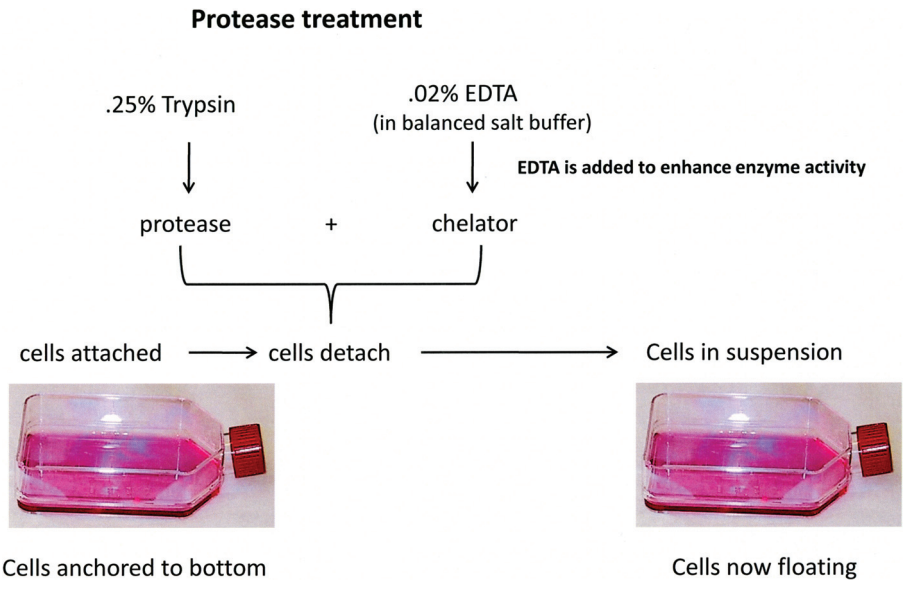

Figure 1.9 The subculture of adherent cell cultures. Trypsin is used to dissociate cells from a monolayer in order to produce a cell suspension that can more readily be diluted and handled.

Cell Quantification

It is important to use a consistent number of cells when freezing stocks or seeding flasks and experimental plates because consistency improves reproducibility by allowing the behavior of the cultures to be more readily maintained and monitored. C2C12 myoblasts will spontaneously fuse when coming into contact with neighboring cells. Thus, the character of the C2C12 culture can change dramatically if fast-fusing cells are allowed to differentiate and fall out of culture. Therefore, it is critical to maintain control over cell density in order to achieve the best experimental results.

(1) While maintaining good sterile technique sample, an aliquot of a cell suspension is obtained as described in Step 5 of "Subculturing".
(2) To this aliquot, add an appropriate volume of Trypan Blue exclusion dye and gently mix. Nonviable cells will become stained blue.
(3) Place a cover slip over the chamber of a hemacytometer and load 10 μL of the sampled cell suspension per side of the chamber.
(4) Place the hemacytometer under a microscope and count the number of viable and nonviable cells found in a defined portion of the grid. Take the mean counts for each cell population and calculate the percentage viability (number of viable cells/total cells) and the concentration of viable cells (number of viable cells × dilution factor × the correction factor based upon the area of the grid) (Fig. 1.10).

Induction of Differentiation and Fusion

The differentiation and fusion of C2C12 myoblasts occurs spontaneously upon the obtainment of cell-to-cell contact. Fusion can be induced by withdrawing serum. This is accomplished by removing the typical growth medium containing 10% fetal bovine serum and feeding the cultures growth medium supplemented with 5% Horse serum (Fig 1.11).

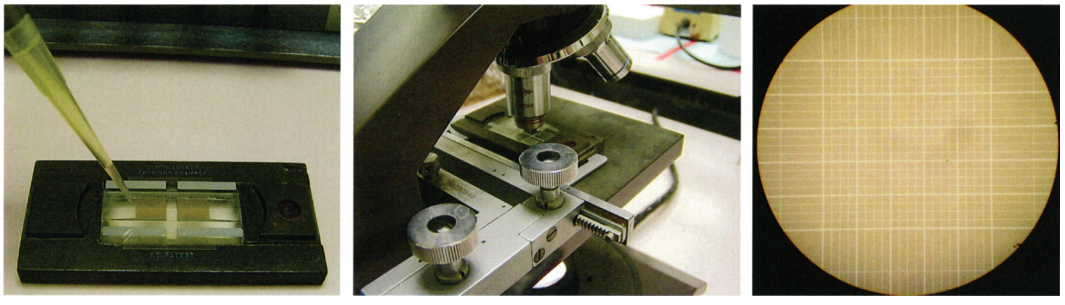

Figure 1.10 Cell quantification using a hemacytometer. The hemacytometer allows cells to be counted within a grid of known surface area. In this way, cell counts can be related to volume allowing the calculation of cell concentration.

Cryopreservation

Cultures selected for cryopreservation should be viable, they should display the expected growth rate and fast-fusing character of C2C12 myoblasts, and they should be free of contamination. Furthermore, preconfluent cultures should be utilized to ensure that they are in the log phase of growth and to maximize the number of fast-fusing myoblasts in the cultures. Freeze medium consists of DMEM supplemented with 20% fetal bovine serum and 5% DMSO (v/v) as the cryoprotectant.

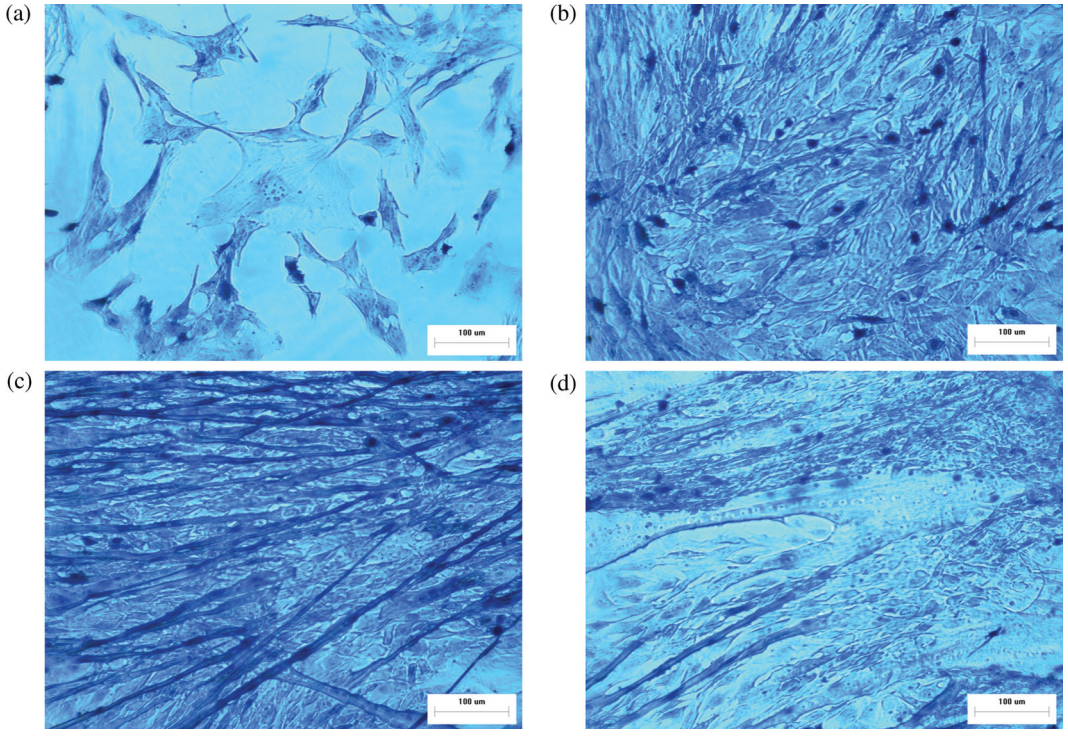

Figure 1.11 Myogenic differentiation of the C2C12 muscle cell line. (a) Subconfluent, proliferative myoblasts approaching 50% confluence. (b) Confluent monolayer of myoblasts. Some spontaneous fusion has given rise to immature myofibers exemplified by an elongated morphology. (c) C2C12 cultures 4 days after serum withdrawal characterized by fully differentiated myotubes. (d) C2C12 cultures 7 days after serum withdrawal. Note the multinucleated nature of mature myotubes.

Inclusion of DMSO prevents crystal formation and dehydration which could negatively impact cell viability. However, DMSO is toxic to C2C12 myoblasts when exposure is prolonged at high concentrations.

(1) Dissociate cells from monolayer to obtain a cell suspension as described in Step 3 of "Subculturing".
(2) Sample a small aliquot to facilitate cell quantification.
(3) Centrifuge the remaining cell suspension at 200 *g* for 5 min to pellet the cells.
(4) Remove the supernatant and re-suspend cells at a concentration of 1–3 $\times$ 10^6 cells per milliliter in freeze medium.
(5) Pipette 1 mL aliquots of cells into labeled cryogenic vials. Label should include cell line name, data, passage number, cell concentration, and the initials of the preparer so that vials can be cross-checked to the appropriate laboratory notebook.
(6) During initial freezing, cultures should be cooled at a uniform rate of -1 to -3°C per minute. This is best accomplished by placing the vials in a Cryo-Safe cooler saturated with isopropanol and stepping down the temperature by placing it an ultra-freezer (-80°C) for several hours before transferring the vials to a cryogenic tank containing liquid nitrogen. Once appropriately frozen, temperature fluctuations should be avoided during storage. Furthermore, vials should be stored in the liquid nitrogen vapor phase to avoid over chilling.
(7) Finally, the location, number, and content of the vials should be recorded in the storage log dedicated for the cryogenic tank being utilized. This allows vials to be quickly retrieved, thus preventing temperature fluctuations in the cryogenic tank.

Thawing Cryopreserved Stocks

Bringing cell stocks back up from cryopreservation is a fairly straightforward process. However, there are some factors to consider which can significantly affect cell viability of newly thawed cultures. When thawing preserved cultures, vials should be warmed to 37°C as quickly as possible by incubation in a water bath for 3–5 minutes. Given DMSO is toxic to C2C12 cells, steps should be taken to wash DMSO out of the cell suspension upon thawing.

(1) Prepare a conical tube containing 10 mL of pre-warmed growth medium for each vial to be thawed.
(2) Wear a laboratory coat and protective eyewear when thawing cells. Occasionally, the lids of cryovials will explode due to pressure differences that can occur during the thawing process. Thus, it is also advisable to place thawing vials in a sealed container during handling whenever possible.
(3) Place vials in a 37°C water bath and thaw stocks as quickly as possible. A slow thaw can dramatically reduce cell viability. Furthermore, large amounts of DMSO can be toxic to C2C12 cells. So, it is necessary to remove the DMSO as quickly as possible.
(4) Once thawed, transfer the vials to a sterile culture hood while thoroughly wiping down each vial with kemwipes saturated with 75% ethanol.
(5) Carefully remove the cap from the vial being mindful to avoid aspirating the contents and to prevent the transfer of cells from one's gloves to inappropriate surfaces. Transfer the cell suspension to the prewarmed growth media in a slow, drop-wise fashion. Then, gently invert the conical tube several times to mix the contents.
(6) Centrifuge the cell suspension at 200 *g* for 5 min to pellet the cells and allow the removal of DMSO when the supernatant is removed.

(7) Re-suspend the cells in 10 mL of growth medium. Provided the cells were frozen down at a concentration of 1–3 x 10^4 cells per millliliter, one vial should sufficiently seed a 75 cm^2 culture flask at a density less than 50% confluence assuming no cell loss. However, a 10–20% loss of viability should be expected. Cells require as much as 4–8 h to become attached. Furthermore, the longer cultures were stored frozen, the greater the expected loss of viability.

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2 Animal Growth and Empty Body Composition

Michael Dikeman

Composition of the Empty Body from Birth to Harvest

It is important for students, researchers, livestock producers, and meat-processing companies to be knowledgeable about the changes in weights and(or) percentages of protein, lipid, and mineral (ash); or muscle, adipose tissue, and bone from birth to market age or maturity. Changes in body composition are related to efficiency of feed nutrient utilization, economics of production, and carcass value. Adipose tissue (fat) deposition is much more costly than muscle deposition. Therefore, it is important to have reference or baseline values for either chemical or physical composition at several times during the lives of animals.

Figure 2.1 illustrates the common sigmoid-shaped growth curve for all meat animals. Weight increases postnatally at an increasing rate until the point of inflection at which time weight begins to increase at a decreasing rate. The rate of weight change is most rapid near the inflection point. As illustrated in Figure 2.2, the rate of weight change can be different for animals within the same species. However, both curves are sigmoid-shaped. Differences in rates of growth can be important economically.

Figure 2.3 shows the weight changes in muscle, fat, and bone as animals grow from birth to maturity. Bone is the most developed of the three tissues at birth and increases the least in weight thereafter. Fat is the least developed at birth and increases slowly initially and then begins to increase at an increasing rate. It can increase at a more rapid rate than muscle as animals approach or pass their optimum harvest endpoint. Muscle is intermediate in development at birth, increases rapidly for several months, and then begins to increase at a decreasing rate. If animals are fed a high-energy diet long enough, the weight of fat can exceed the weight of muscle.

Figure 2.4 shows the changes in *percentages* of muscle, fat, and bone as animals mature. Because bone is the most developed tissue at birth, its percentage begins to decrease at a moderate rate until after weaning age and then the percentage decreases rather slowly. Percentage of muscle is greatest initially but it also decreases in percentage from a few months of age until maturity, whereas the percentage of fat increases in a near-linear fashion beginning after a few months of age. For most species, the harvest endpoint should occur when sufficient fat has been deposited to attain optimum meat quality and carcass meat yield.

Figure 2.5 shows the changes in amounts of water, fat, protein, and ash as animals grow to maturity. The increase in the amount of ash is rather subtle to a weight of about 700 kg in cattle. The

The Science of Meat Quality, First Edition. Edited by Chris R. Kerth.

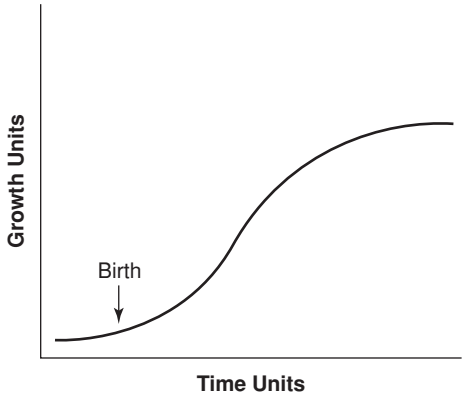

Figure 2.1 Typical growth curve. Reprinted with permission from Boggs et al. (1998). Copyright © 1998 by Kendall/Hunt Publishing Company. Reprinted by permission.

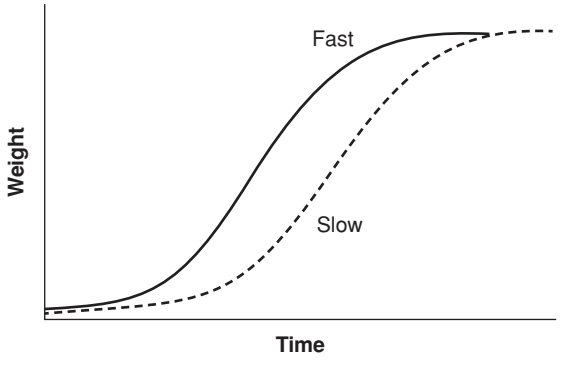

Figure 2.2 Growth curves of fast and slow-growing animals. Reprinted with permission from Boggs et al. (1998). Copyright © 1998 by Kendall/Hunt Publishing Company. Reprinted by permission.

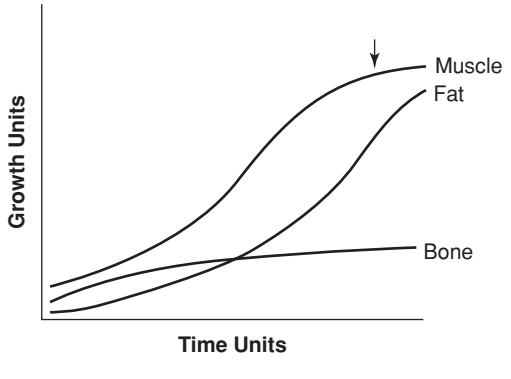

Figure 2.3 Postnatal growth curves of bone, muscle, and fat. Reprinted with permission from Boggs et al. (1998). Copyright © 1998 by Kendall/Hunt Publishing Company. Reprinted by permission.

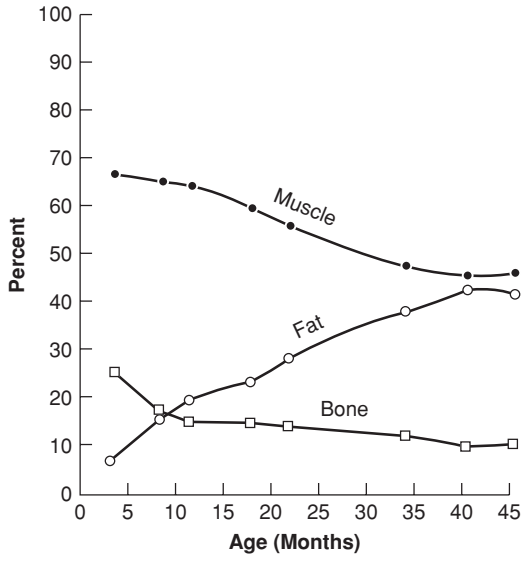

Figure 2.4 Changes in the percentage of bone, muscle, and fat in beef carcasses during growth. Adapted from Moulton et al. (1922). Courtesy of W.H. Freeman and Company.

increase in protein is rather linear, whereas the amount of fat increases at a rapid rate after weaning age when cattle are fed a moderate- to high-energy diet.

Wagner et al. (1999) measured body composition of 319 gilts and barrows at 8 different weights from 25 to 152 kg live weight. Nonlinear growth functions accounted for the greatest amount of variation in empty body protein, lipid, moisture, and ash mass. Empty body is defined as the sum of carcass, head, and viscera with contents of the gastrointestinal tract removed. Differences existed

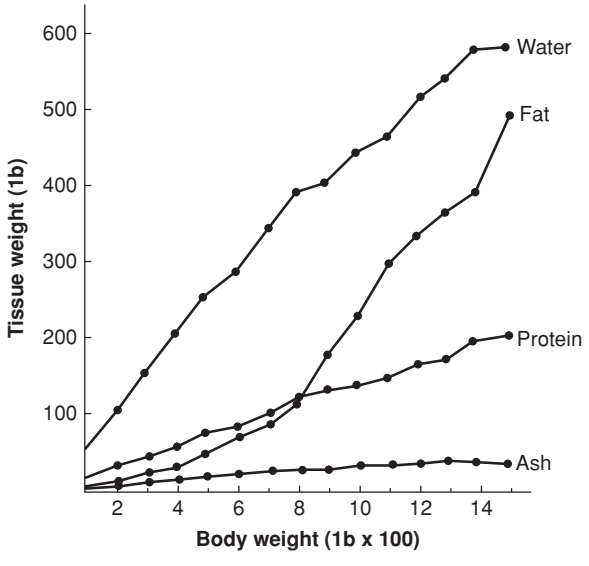

Figure 2.5 Chemical component changes with empty body weight of growing steers. Adapted from Berg and Butterfield (1976)

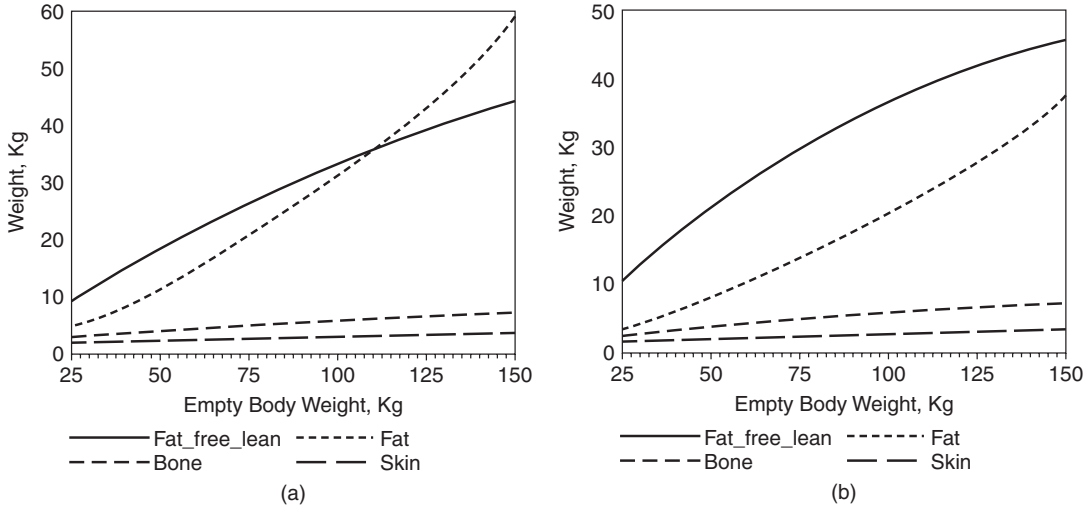

Figure 2.6 Growth of carcass fat-free lean, fat, skin, and bone. Carcass fat-free lean, skin, and bone mass data were fitted to nonlinear functions of empty body weight. Carcass fat mass data were fitted to an exponential function of empty body weight. (a) Barrows and (b) Gilts. Adapted from Wagner et al. (1999).

between gilts and barrows for nearly all components, although the shapes of the growth curves were similar (Figs. 2.6a, 2.6b, 2.7a, and 2.7b). The point at which mass of carcass fat exceeded that of fat-free lean occurred at a lesser body weight in barrows (approximately 110 kg) than in gilts (approximately 140 kg). This data set should be an extremely useful one regarding changes in empty body composition of gilts and barrows over a wide weight range.

Table 2.1 shows overall means of component weights of the empty body of barrows and gilts combined and harvested over a wide weight range. These data are valuable reference points for changes of body components.

Table 2.2 shows the developmental changes in carcass characteristics of barrows and gilts separately over a wide weight range. Note that dressing percentage increases considerably from 100 to 152 kg and increases more rapidly for barrows than for gilts. Differences between barrows and gilts in 10th rib fat depth begin to become larger after 64 kg, whereas the gender difference in last rib backfat does not occur until about 114 kg. Gilts have an advantage in longissimus muscle beginning early in life.

Means for mass of protein, lipid, moisture, and ash of barrows and gilts are shown in Table 2.3. There is a much greater difference in percentage of lipid than there is for protein. Percentages of ash are not much different between barrows and gilts.

Differences between barrows and gilts in mass of lean, fat, and other soft tissue are shown in Table 2.4. There is more difference in percentage of lean than there is for fat.

Researchers, students, industry personnel, and livestock producers might have a need to determine or predict the chemical or physical composition of breeding animals, market animals, carcasses, or major portions of carcasses. The need or interest in knowing compositional differences might include the goal of genetic improvement of the composition of livestock; of improvement in the efficiency of converting nutrients into protein, lipid, and minerals; of evaluating the effects of nutritional regimens, including metabolic modifiers, on the proportions of muscle, adipose tissue,

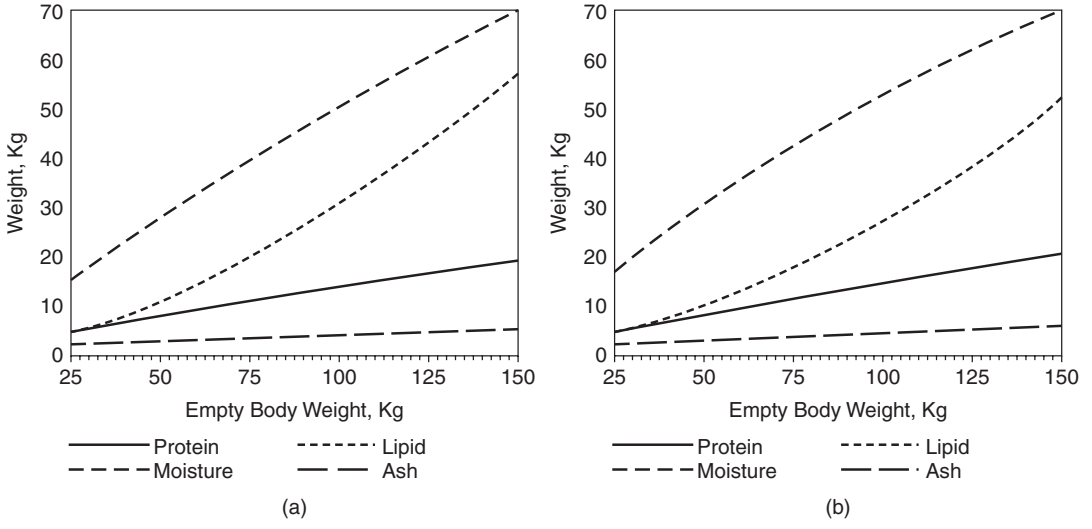

Figure 2.7 Growth of empty body protein, lipid, moisture, and ash. Empty body protein and moisture mass data were fitted to nonlinear functions of empty body weight. Ash mass data were fitted to allometric functions. Empty body mass data were fitted to an exponential function of empty body weight. (a) Barrows and (b) Gilts. Adapted from Wagner et al. (1999).

and bone; or determining value differences in carcasses or major portions of carcasses. Sometimes, the interest might be empty body composition of animals, but most commonly the interest is in carcass or major portion composition.

Chemical Composition

When the weights and/or proportions of protein, lipid, and mineral are desired, the reference and 'gold standard' method is to grind the entire carcass, including bone and heavy connective tissue, thoroughly mix and sample the tissue, and then conduct analyses for protein, lipid, and ash. Obviously, this will require a very large grinder but only some laboratories have them. The

Table 2.1 Overall means of component weights of the empty body

Weight group (kg)	*N*	Slaughter weight (kg)	Empty body weight[a] (kg)	Warm carcass weight (kg)	Head weight (kg)	Viscera weight[b] (kg)
25	41	26.0	24.4	17.7	1.9	4.8
45	40	42.9	39.7	30.0	2.8	6.9
64	44	63.3	57.7	44.9	3.8	9.3
84	40	82.4	75.5	59.7	4.7	11.1
100	38	98.0	90.1	71.8	5.5	12.8
114	41	112.2	103.6	83.5	5.9	14.2
129	38	127.0	118.7	96.4	6.6	15.7
152	37	149.6	139.3	113.6	7.5	18.3

[a]Empty body is defined as the sum of the arm carcass, head, and viscera components.
[b]Viscera is a composite sample of the visceral organs, blood, and leaf fat.

Table 2.2 Development changes of carcass characteristics (least squares means)

		Dressing percentage (%)		10th rib 3/4 fat depth (cm)		Loin muscle areas (cm^2)		Last-rib backfat thickness (cm)		Carcass length (cm)	
Weight group (kg)	Genetic population	Barrows	Gilts	Barrows	Gilts	Barrows	Gilts	Barrows	Gilts	Barrows	Gilts
25	Mean	68.09	68.31	0.91	0.92	12.43	12.59	0.83	0.83	53.10	52.90
45	Mean	68.55	70.83	1.43	1.32	17.32	20.32	1.34	1.35	61.64	62.09
64	Mean	70.09	70.84	1.97	1.73	23.60	26.38	1.89	1.88	69.26	69.27
84	Mean	71.93	73.06	2.49	1.99	29.93	31.49	2.29	2.07	73.44	75.42
100	Mean	72.55	73.92	2.80	2.52	29.14	34.97	2.66	2.71	78.38	78.97
114	Mean	74.26	74.59	3.26	2.78	33.48	38.50	3.27	2.78	81.38	80.50
129	Mean	75.53	76.23	3.60	3.05	33.31	40.88	3.56	3.03	84.19	84.45
152	Mean	76.18	75.66	4.05	3.44	40.24	45.45	4.00	3.27	86.40	88.05
Average SE		0.72		0.20		1.75		17		0.90	
Effects[a]		W***, W × S*		W***, W × G*, W × S**		W***, W × G†, W × S**		W***, W × G*, W × S***		W***, W × G*, W × S*	
Development[b]		Q	Q	L	L	Q	Q	Q	Q	Q	Q

[a]W, weight group; S, sex; G, genetic population; W × G, weight group by genetic population interaction; W × S, weight group by sex interaction.

[b]L, development of the characteristic was linear ($p < .05$) with respect to empty body weight; Q, development of the characteristic was quadratic ($p < .05$) with respect to empty body weight.

†$p < .10$; *$p < .05$; **$p < .01$; ***$p < .001$.

Table 2.3 Mass of protein, lipid, moisture, and ash in the empty body (least squares means)

		Protein (kg)		Lipid (kg)		Moisture (kg)		Ash (kg)	
Weight group (kg)	Genetic population	Barrows	Gilts	Barrows	Gilts	Barrows	Gilts	Barrows	Gilts
25	Mean	3.37	3.36	3.23	3.09	16.28	16.40	0.80	0.80
45	Mean	5.64	6.04	6.75	6.35	24.80	25.61	1.34	1.29
64	Mean	8.14	8.30	11.98	11.50	34.44	34.79	1.92	1.92
84	Mean	10.43	11.00	19.39	17.27	42.04	43.86	2.41	2.55
100	Mean	12.02	12.53	26.17	24.16	47.62	48.93	2.59	2.90
114	Mean	13.39	14.28	33.34	28.82	52.02	54.71	3.05	3.29
129	Mean	14.85	16.57	41.89	35.65	56.28	60.81	3.44	3.73
152	Mean	17.80	18.94	50.70	45.52	64.63	67.75	4.18	4.55
Average SE		0.42		1.29		1.04		0.19	
Effects[a]		W***, W × G*W × S**		W***, W × G*, W × S***		W***, W × S***		W***, W × S†	
Development[b]		Q	Q	Q	Q	Q	Q	L	L

[a]W, weight group; S, sex; G, genetic population; W × G, weight group by genetic population interaction; W × S, weight group by sex interaction.

[b]L, development of the characteristic was linear ($p < .05$) with respect to empty body weight; Q, development of the characteristic was quadratic ($p < .05$) with respect to empty body weight.

†$p < .10$, *$p < .05$, **$p < .01$, ***$p < .001$.

Table 2.4 Mass of dissected carcass lean, dissected carcass fat, and other soft tissue (least squares means)

		Dissected carcass lean (kg)		Dissected carcass fat (kg)		Other soft tissue (kg)	
Weight group (kg)	Genetic population	Barrows	Gilts	Barrows	Gilts	Barrows	Gilts
25	Mean	7.83	7.80	1.73	1.75	2.71	2.71
45	Mean	13.089	13.94	3.93	3.66	5.20	5.15
64	Mean	18.69	19.73	7.68	7.01	8.37	8.04
84	Mean	24.23	25.79	11.66	9.88	11.55	11.50
100	Mean	26.96	29.77	16.33	13.92	14.64	14.10
114	Mean	31.07	34.59	19.53	16.32	17.13	17.26
129	Mean	33.45	38.46	25.07	19.54	21.13	20.66
152	Mean	39.50	43.58	30.41	25.70	25.21	23.83
Average SE		1.1		1.2		0.53	
Effects[a]		W***, W × G** W × S†		W***, W × G**, W × S**		W***, W × S†	
Development[b]		Q	Q	Q	Q	Q	Q

[a]W, weight group; S, sex; G, genetic population; W × G, weight group by genetic population interaction; W × S, weight group by sex interaction.
[b]L, development of the characteristic was linear ($p < .05$) with respect to empty body weight; Q, development of the characteristic was quadratic ($p < .05$) with respect to empty body weight.
†$p < .10$, *$p < .05$, **$p < .01$, ***$p < .001$.

other option is to physically and completely remove all soft tissue from the bones and grind and chemically analyze the soft tissue for protein, lipid, and ash. Both of these procedures are very expensive, primarily because of the loss of meat value. Tissue from complete carcass grinding and chemical analyses cannot be sold for human consumption because of the bone particles in the tissue. There would be less economic loss when only the soft tissue from carcasses is ground and analyzed, but the value of the salvaged tissue will still be dramatically reduced because no steaks, chops or roasts can be sold and the composition of the soft tissue might contain more lipid than is marketable unless it is mixed with other lean tissue.

The alternative to conducting chemical or physical composition of whole carcass sides is to conduct chemical analyses of major portions or cuts of carcasses for which accurate prediction equations have been developed. For beef carcasses, chemical analyses of the 9-10-11th rib section are relatively accurate for predicting carcass chemical components.

Physical Separation

When proportions of muscle, adipose tissue, and bone are of primary interest, the reference method is physical separation of a carcass or carcass side. Obviously, this is an extremely labor-intensive procedure and, thus, very expensive. In addition, there can be significant errors when subcutaneous and intermuscular adipose tissues are not carefully and completely removed from muscle. When the lipid composition of carcasses is quite variable, the muscle tissue should also be chemically analyzed for protein, lipid, and moisture because differences in percentages of intramuscular lipid can affect the weights of muscle. It might be important to conduct moisture and lipid analysis of the separated adipose tissue because of differences in physiological maturity of adipose tissue. Complete physical separation accompanied with chemical analyses would be more closely related to economic value than chemical analyses of entire carcass sides because weights of major muscles with high economic value can be obtained. Because of the extreme labor costs and very small number of carcasses that can be done per hour, physical separation, with or without chemical analyses, would only be recommended in a limited number of research projects.

The alternative to total carcass side physical separation would be physical separation, with or without chemical analyses, of major portions or primal cuts. For predicting beef carcass composition, the 9-10-11th rib cut has been used frequently over the past 50 years. There could be differences in accuracy of using 9-10-11th rib composition when considering the differences in bone development in young cattle versus mature beef cows. Even so, the 9-10-11th rib procedure has been and is used quite frequently for predicting physical or chemical composition of beef carcasses. Certainly, physical separation of a major portion or cut of a carcass would be much more economical than separation of carcass sides.

The Hankins and Howe (1946) procedure for removing the primal rib and then the 9-10-11th rib section is described here. The forequarter is removed from the hindquarter between the 12th and 13th ribs by a cut that crowds the 12th rib its full length. A metal ruler at least 0.7 m long and a T-square are needed for removing the rib. Point A is the “point” (ventral edge) of the body of the split thoracic vertebra and “point” B is the cartilage or “button” end of the 13th rib that angles toward the sternum. The distance between A and B is measured and the distance from point A to point C on the ruler is to be 61.5% of the A to B distance. At point C on the ruler, a perpendicular is projected by means of the T-square or carpenter’s square to the external surface of the carcass (point D) where a mark is made. The cut to remove the primal rib is to be perpendicular to the external surface of the carcass. The primal rib is then removed between the 5th and 6th ribs by a cut that

crowds the 5th rib all the way. The original Hankins and Howe (1946) procedure describes how the shank, brisket, and plate are to be removed by cutting from a point on the 12th rib that is 66.6% of the length of the rib from the backbone to a point just dorsal to the lateral condyle of the humerus. However, because the primal rib often has to be removed from a hanging side in a commercial plant without removal of the shank, brisket, and plate, an alternative procedure is recommended, which is to mark the same distance from the backbone to the 6th rib as the distance from the backbone marked on the 12th rib. This results in a slightly longer primal rib than the original procedure, but should not affect precision of the data.

The 9-10-11th rib section is removed by cutting with a large knife perpendicular to the outer surface and vertebrae and crowding the posterior edge of the 8th and 11th ribs through the backbone. The cuts should be as parallel as possible.

It should be pointed out that the equations developed by Hankins and Howe (1946) were developed from cattle that were quite different in biological type than cattle produced at the time of this publication. Even so, it is used quite frequently for predicting either chemical or physical components of beef carcasses. It should be very good for determining certain treatment or breed differences among animals. It may not be adequate for nutritional balance studies.

Magnetic Resonance Imaging

A magnetic resonance imaging (MRI) analysis relies on the volume measurements of specific regions of muscle and adipose tissue (Mitchell et al., 2001). It is a non-invasive, *in vivo* body composition analysis ranging from a volumetric measurement of a specific tissue or organ to prediction of total body fat and lean content. Mitchell et al. (2001) evaluated the accuracy of MRI to predict composition of light-weight pigs ranging from 6.1 to 97.2 kg. The best prediction equation for percentage of total body fat was obtained using the fat volume from the 10 cm section of longissimus muscle and the fat/muscle ratio from the 15 cm section of the ham ($R^2 = 0.90$). The best prediction equation for percentage of total body protein was obtained using a combination of the volumes (as a percentage of BW) of jowl fat, backfat, shoulder muscle, and ham muscle ($R^2 = 0.62$). The combination fat volume from the 10 cm section of longissimus muscle, the fat/muscle ratio from the 15 cm section of the ham, and the lean volume percentage from the 15 cm section of ham provided the best prediction of the percentage of total body lean ($R^2 = 0.88$).

Collewet et al. (2005) evaluated X-ray computer technology, vision techniques, and MRI in pigs for predicting carcass composition. The best standard error of prediction using a linear regression with the dissection of the left half carcasses was 586 g and 1.10% for lean meat weight and lean meat percentage, respectively. These authors stated that MRI could be used in place of full dissection for authorizing and monitoring classification equipment of pig carcasses. In comparing the research of Mitchell et al. (2001) and Collewet et al. (2005), equations were based on different kinds of traits. The Mitchell et al. (2001) equations utilized fat/muscle ratios, volumes of components, and image tracings. The equation of Collewet et al. (2005) used only images of cross sections. These differences suggest that the combination of traits that resulted in the best prediction equations might not be repeatable from one set of pigs to another. These contrasting results suggest that equations might need to be developed for each set of pigs or carcasses rather than use a standard prediction equation for each.

MRI equipment is very expensive and there is a high level of training required for using this technology. The technology might not be practical for industry or most institutional research. For these reasons, the technology would not be available or accessible to most industrial companies or institutions.

X-Ray Computed Tomography Scanning

Holm et al. (2009) used computed tomography (CT) as a selection tool for carcass composition of intact male swine. They stated that CT was more accurate than dissection for meat and fat content. In addition, they stated that the heritability estimate for lean meat content was 0.57. The transformation of CT images to useable data for genetic evaluation was performed by medical image analysis and proprietary computer programs.

Navajas et al. (2008) evaluated X-ray CT of individual primal joints to predict beef carcass composition of a small sample of 7 Angus and 15 Limousin steers. The R^2 values for weights of tissues in the primal cuts were high (0.91 for fat; 0.96 for bone, and 0.99 for muscle). They did not report R^2 values for percentages of the tissues. These authors stated that CT scanning is especially valuable for assessing carcass composition of large numbers of animals in genetic selection programs. They also stated that it should be noted that the tissue thresholds estimated in this study would need to be independently validated in other data sets. In addition, it should be recognized that the sample size in this study is small and that the accuracy of predicting carcass composition of heifers and/or other biological types of cattle might be different.

Lambe et al. (2008) used live weight, subjective scores of condition and conformation, live animal video image analysis, ultrasound, and X-ray CT scanning in live Texel lambs (240) and Blackface lambs (233) to predict carcass composition. At harvest, one side of the carcass was fully dissected to determine muscle and fat weights. Predictors derived from CT alone accounted for a high proportion of the variance in dissected fat and muscle weight in Texel lambs (adjusted $R^2 = 0.80$), as well as intramuscular fat content in the loin (0.60), but lower proportions in Blackface lambs. Adding traits measured by other *in vivo* methods (video image analysis and ultrasound) increased prediction accuracies (adjusted R^2) by up to 0.26, depending on trait and data set.

Vester-Christensen et al. (2009) evaluated CT of 299 pork carcass sides to predict composition of a subsample of 29 carcasses for physical dissection. They reported that, on average, CT scanning identified 1227 g more meat, 968 g less fat, and 225 g less bone in the carcasses than manual dissection. On a percentage basis, CT scanning resulted in 3.07% more meat, 2.49% less fat, and 0.58% less bone. They reported an R^2 value of 0.9994 using a "leave-one-out cross validation" procedure. These authors stated that disregarding the fixed costs related to the purchase of a CT scanner and installing it in a trailer, the lower costs using CT is a considerable advantage compared to manual dissection. If only the maintenance of the scanner is taken into account alongside the salary of the operators, a CT-based LMP costs less than half that of manual dissection.

The main limitation to the use of X-ray CT scanning is the cost of the equipment and the required technical expertise for operation and interpretation of the data, which will greatly limit the number of institutions and corporations who can use this technology. The cost of the equipment likely will range from US$200,000 to US$250,000. One institution in the European Union charges US$75 per sheep scan, which would be cost prohibitive except for elite seedstock producers.

Near-Infrared Reflectance

Near infrared (NIR) refers to the range within the electromagnetic spectrum with wavelengths from approximately 760 to 2500 nm, slightly longer than visible light (approximately 400–760 nm) (Clarke, 2004). The majority of NIR instruments were developed as laboratory instruments and not for online implementation. Little sample preparation is required to use NIR, the technology is rapid, and it is nondestructive. This equipment is expensive, requires a high level of training for its use,

and it does not consider value differences among muscles or primal cuts. Very few institutions and industrial companies would have access to this technology.

Total Body Electromagnetic Conductivity (TOBEC)

The total body electromagnetic conductivity (TOBEC) system relies on the principle that lean meat conducts electricity better than fat. Research has been published on the accuracy of the TOBEC system in predicting the composition of live lambs, carcasses of pork and lamb, and beef hindquarters (Forrest et al., 1991). Higbie et al. (2002) evaluated the accuracy of the TOBEC system to predict total fat-free lean weight and total fat weight in pork carcasses and their hams from 32 gilts representing a range in weight, muscling, and fatness. Physical dissection and chemical analyses were used to determine fat-free lean and fat weights. They reported an R^2 value of 0.91 when using the peak value obtained from scanning the left carcass side in the equation to predict weight of fat-free lean. They did not report an equation for predicting percentage of fat-free lean. The sample size of only 32 gilts is somewhat weak to place much confidence on the equation that they reported.

Berg et al. (1997) evaluated the TOBEC system and bioelectrical impedance for their accuracy in predicting total dissected lean in 106 lamb carcasses. The best TOBEC equations for predicting weights of total dissected lean and fat-free lean on warm carcasses both had R^2 values of 0.88 and 0.88, respectively. Both of these equations included carcass weight. The best equations for predicting percentages of total dissected lean and fat-free lean had R^2 values of 0.72 and 0.78, respectively. The R^2 values for equations based on chilled carcass TOBEC readings were 0.62 and 0.62, respectively. The R^2 values for bioelectrical impedance measured on chilled carcasses were slightly lower than those for TOBEC measurements on chilled carcasses.

Wishmeyer et al. (1996) evaluated the TOBEC system coupled with weight and other lamb measurements on 47 Rambouillet live lambs for predicting chemical composition based on whole-animal ground samples. Lambs ranged in weight from 29.5 to 63.5 kg. Reasonably reliable prediction equations were obtained for dry matter, crude protein, ether extract, and fat-free mass ($R^2 > 0.66$). However, the TOBEC scan responses contributed little to the model sum of squares, whereas body weight accounted for the majority of the model sum of squares. These authors concluded that the TOBEC system was not a reliable tool for predicting chemical composition of live lambs.

Gwartney et al. (1994) evaluated the TOBEC system to predict lean content of beef carcasses and cuts of 100 steers and heifers representing a broad range in fatness and live weight. Prediction equations for percentages of side lean based on hindquarter scans from steers and heifers resulted in R^2 values of 0.75 and 0.80, respectively. Prediction accuracy when scanning forequarters was slightly lower than when scanning hindquarters.

It can be concluded that the TOBEC system is not accurate enough for predicting composition of live lambs but is reasonably accurate for predicting carcass composition. Few institutions would have this system for research and it may not be rapid enough for large-scale processing plants.

Dual-Energy X-Ray Absorptiometry

Dual-energy X-ray absorptiometry (DXA) is an alternative to computer-assisted tomography or MRI for predicting pork carcass composition. It is lower in cost and easier to use but is a slow technology. Marcoux et al. (2005) studied 95 gilt carcasses from three genetic lines varying widely in composition. They separated pork carcass sides into four primal cuts before scanning with

a DXA instrument that was calibrated for estimating human body composition. Although these authors reported an R^2 value of 0.85 for predicted weight of dissected lean, the mean values between dissected lean and DXA-predicted lean were quite different. Predicted values were approximately 50% greater than actual dissected lean. This was partially explained by the fact that the lean in the front shank, jowl, side ribs, and neck bones was not dissected, whereas lean from those parts were included in the DXA values. Even so, there was still a difference for which there was no account. Correspondingly, the weight of dissected fat (skin included) was approximately 50% greater than DXA-predicted values.

Marcoux et al. (2005) stated that correcting for the different genetic lines was not necessary to improve the accuracy of prediction. The prediction equation for fat weight resulted in a modest R^2 value of 0.70. The authors did not present equations for predicting percentages of lean or fat; only weights.

Pearce et al. (2009) used DXA to predict both live and carcass composition of Merino wethers. The wethers were held off feed and water for 24 h prior to anesthesia and DXA scanning. Scan times were approximately 2 min per animal or carcass. Chemical composition involved grinding all bone, muscle, and fat. The relationship between chemical lean (protein + water) was highly correlated with DXA carcass lean ($R^2 = 0.90$) but only moderately with DXA live lean ($R^2 = 0.72$). However, because the predicted live lean mass was the sum of muscle, organs, blood, and stomach contents, predicted chemically determined live lean was 39.9 $\pm$ 0.87 kg compared with 15.4 $\pm$ 0.20 kg carcass lean. This illustrates that high correlation values do not necessarily relate to accuracy in predicting actual amounts of lean, fat, or bone. When live weight was included in the DXA equation to predict carcass lean, the correlation was improved ($R^2 = 0.82$). The relationship between chemical fat was moderately correlated with DXA carcass fat ($R^2 = 0.86$) and DXA live fat ($R^2 = 0.70$). DXA carcass lean and DXA carcass fat, with the inclusion of carcass weight in the regression, significantly predicted boned-out muscle ($R^2 = 0.97$) and fat ($R^2 = 0.92$). The use of DXA live lean and DXA live fat with the inclusion of live weight to predict boned-out muscle ($R^2 = 0.83$) and fat ($R^2 = 0.86$) weight, respectively, was moderate. Prediction accuracies dropped when percentages of lean, fat, and bone were predicted.

Pearce et al. (2009) concluded that the prediction accuracy for predicting lean and fat is clearly better for carcasses than for live animals. However, they stated that very specific prediction equations were used that were highly specific to breed and weight ranges used and that the predictive models may not transport well to other breeds or greater weight ranges. Yet, they state that the potential to scan live animals and obtain an accurate *estimate* (my emphasis) of both carcass composition and boned-out muscle weights was identified as a clear benefit. Therefore, their data do not totally support the statement that it is accurate for predicting live composition.

Dunshea et al. (2007) used DXA to determine carcass composition of 60 half carcasses from ewes and wethers of five genotypes. Both lean tissue and fat tissue weight and percentage were predicted with high accuracy ($R^2 = 0.985$ for lean weight and 0.937 for percentage, and 0.988 for fat weight and 0.942 for percentage, respectively). These authors stated that it is important to note that, with the exception of total tissue mass, the regression equations all differ from unity and appropriate predictive equations need to be used to quantitatively predict half carcass composition. These authors also developed prediction equations using HCW, GR fat, and eye muscle depth and reported R^2 values of 0.966 and 0.842 for lean tissue weight and percentage, respectively; and 0.972 and 0.876 for fat tissue weight and percentage, respectively. Although these latter equations are quite accurate, the authors state that the DXA offers better precision for research purposes but may have application for commercial application in the future. Ponnampalam et al. (2007) reported that DXA technology was more accurate for predicting fat than muscle.

Mercier et al. (2006) tested DXA to predict carcass composition of 140 male and female Dorset and Suffolk lambs of four weight classes. The prediction of half carcass dissected fat percentage was rather weak with an R^2 of only 0.70. The R^2 obtained when predicting dissected lean was 0.93. The authors concluded that DXA is an effective technology for predicting the amount of lean and fat in lambs and amount of lean total weight and fat in lamb carcasses.

Results from these studies do not suggest that the technology is practical because few institutions or processing companies would have a DXA instrument. When researchers determine that they need to utilize this technology, they would need to consult with the institutions that have the instrumentation and technology required for operation.

Video Image Analysis

Hopkins et al. (2004) used the VIAScan® system developed by Meat and Livestock Australia to predict composition of lamb carcasses. When 8 VIAScan® measures were used to predict lean meat yield, the R^2 was only 0.52 (residual standard deviation, RSD = 2.17%). These results are not very supportive for recommending VIAScan® use for predicting lamb carcass composition.

Shackelford et al. (1998) used a video imagine camera system to scan the 12th rib cross section of beef carcasses from 66 steers and heifers to develop an equation for predicting the percentage of boneless retail product (retail cuts with all surface fat removed plus lean trim containing 20% fat). They found that image analysis accounted for 77% of the variation in percentage of retail product by itself, and 88% when combined into the best five-variable equation including other variables. They stated that weight of retail product can be predicted more precisely by multiplying predicted percentage of retail product by carcass weight than by predicting weight of retail product directly. When applying the best five-variable equation from Shackelford et al. (1998) by multiplying by carcass weight, they reported that they found 95% accuracy for predicting weight of retail product. These results suggest that percentage of retail product could be predicted with relatively high accuracy by meat-processing plants with video image analysis equipment and that such data could be very valuable for making breeding decisions or detecting differences due to treatments when sample sizes are sufficiently large. Cost of this instrumentation would be significantly less than for DXA, NIR, or X-ray CT.

Brady et al. (2003) evaluated the lamb vision system (LVS) coupled with carcass weight to predict both boneless and bone-in saleable meat yields and subprimal yields of lamb carcasses. The LVS + HCW equation resulted in an R^2 value of 0.60 for percent boneless saleable meat yield and R^2 = 0.58 for percent boneless subprimal yield. Interestingly, the prediction equation for percent fat yield had a higher R^2 value of 0.74. These R^2 values were only slightly better than expert graders determining USDA yield grades to the nearest tenth. When either longissimus muscle area, fat thickness, or both were evaluated by an expert grader, and included in a prediction equation, the prediction R^2 values increased by only 3–5%. Generally, it is not possible to obtain lamb carcass longissimus muscle area in commercial processing plants. Because it is likely that most institutions will not have LVS equipment and because the LVS + HCW prediction system only accounts for about 60% of the variation in percentage of saleable meat or subprimal yields of lamb carcasses, this system is not recommended for research purposes. These authors stated that the system could be used as an objective means for pricing carcasses in a value-based marketing system.

Cunha et al. (2004) applied the LVS prediction equations developed by Brady et al. (2003) to 149 different lamb carcasses and reported R^2 values of 0.56 for percentage of bone-in saleable meat yield, 0.46 for percentage of bone-in subprimal yield, and 0.62 for percentage of fat yield. These R^2

values were not quite as accurate as those in which expert graders determined USDA yield grade to the nearest tenth. Equations developed by Cunha et al. (2004) on their 149 lambs resulted in R^2 values of 0.68 for percentage of bone-in saleable meat yield, 0.62 for percentage of subprimal yield, and 0.74 for percentage of fat. These authors stated that the system could be used as an objective means for pricing carcasses in a value-based marketing system. The results of Cunha et al. (2004) further suggest that the LVS is not recommended for research purposes when more than bone-in trimmed saleable meat yield is of interest.

Additionally (Hopkins et al., 2004), R^2 values are not accurate enough for most research purposes but might be fine for commercial meat yield prediction automatically in commercial value-based marketing systems.

Farrow et al. (2009) incorporated video images of the 12–13th rib interface of beef carcasses with carcass traits used in calculating USDA yield grades. They reported that accuracy of predicting saleable meat yield was improved relative to current measures used in the USDA yield grade equation (R^2 of 0.70 vs. 0.63, respectively).

Video image systems are being used in numerous beef-, pork-, and lamb-processing plants in Europe and the United States for determining value differences in carcasses. Most institutions would have access to this technology when collecting data in a commercial processing plant that has the instruments.

^{40}K Liquid Scintillation Counter

Domermuth et al. (1976) compared Electronic Meat Measuring Equipment (EMME) with a ^{40}K 2-pi configuration liquid scintillation counter as indirect means of estimating the lean body composition of live pigs. They reported that either the EMME or ^{40}K counter in combination with shrunk body weight were useful in predicting the weight of carcass protein ($R^2 = 0.83$ and 0.69, respectively). However, shrunk body weight had R^2 values at least as good for predicting weight of carcass protein or water. Equations for predicting percentages of protein and water had rather low R^2 values.

Carr et al. (1978) evaluated a ^{40}K counter for predicting carcass composition of 30 Yorkshire and 70 Hampshire barrows over a wide range of live weights. Equations involving live weight, ^{40}K counter, and Duncan Lean Meter backfat probe accounted for 98.2% and 96.3% of the variation in Yorkshires and Hampshires, respectively.

Lohman et al. (1966) used ^{40}K counter to predict composition of 42 steers and reported that 88% of the variation in body lean determined from chemical analysis was accounted for in their best equation. Standard errors of estimate were actually lower when evaluating the steers alive than when evaluating their carcasses.

Frahm et al. (1971) evaluated ^{40}K for predicting muscle in 40 yearling beef bulls. Their prediction equation accounted for 86% of the variation in weight of fat-free lean with no difference between the equation using ^{40}K counter only or in conjunction with live weight.

Rider et al. (1981) evaluated the ^{40}K counter for predicting the composition of 47 yearling British bulls. They reported that an adjusted net count and weight were used in an equation that accounted for 86.3% of the variation in carcass lean. They did not report an equation for predicting percentage of lean. All three of these studies report R^2 values of about 86% for predicting weight of lean, whereas most did not report equations and R^2 values for predicting percentages of lean.

The major limitation to the use of this technology is that there are only two large units in the United States and there has been almost no research conducted using those units for more than a decade. Therefore, this technology is not an option for nearly all institutions and industry.

Dilution Techniques

Mohrmann et al. (2006) described the deuterium dilution technique (DT) as a capable non-invasive method to measure body composition based on body water content. It is a procedure that has no adverse effects on animals or employees. These researchers conducted an experiment with 440 pigs ranging in weight from 20 to 140 kg. Deuterium oxide was applied with a small amount of food provided to pigs after 12 h without access to food. Blood samples were then taken from *Vena jugularis* about 5–6 h after application. Total body water was determined by deuterium space in blood with the following equation:

$$\mathrm{TBW_{D2O}} = \frac{\mathrm{D2O_{fed}(g)}}{\mathrm{D2O\ blood(ppm)}} - \frac{100{,}000}{\mathrm{BW}}(\mathrm{kg}).$$

For the entire empty body (without content of the gastrointestinal tract and bladder), allometric prediction equations to predict body composition from empty body water content measured by the DT were derived from chemically analyzed serial slaughtered pigs (20, 30, 60, 90, 120, 140 kg). The developed equations were correlated with contents of body water (0.92), fat-free substance (0.88), and protein in fat-free substance (0.82). In that study, the correlations of fat tissue content, fat tissue mass, and lean tissue measured by MRI showed allometric relationships to lipid content, lipid mass, and protein mass determined by DT which were 0.98, 0.87, 0.98, respectively. Protein and ash proportions do not change much over the weight range of 20–140 kg, whereas the proportion of water decreased and the proportion of lipid increased.

Earlier studies by Shields et al. (1983) with pigs; Wuthier and Stratton (1957) with cattle; Kock and Preston (1979) with cattle; and Arnold et al. (1985) with cattle demonstrated that live animal empty body composition could be predicted with relative high accuracy using deuterium oxide dilution techniques. However, this procedure has almost no meat-processing industry application, requires considerable training, and does not consider value differences among muscles. It would seem to be a basic research technique that has had limited application. It does have the advantage, however, that it is nondestructive for live animal composition assessment.

Ultrasound Technology

Ultrasound technology has good potential for use in live animals to predict body or carcass composition. It is not logical to use it for carcasses to predict composition when actual carcass measurements can be used. For carcasses, ultrasound data would merely be "predictors of predictors" of carcass composition.

Johnson et al. (2004) published results of an extensive study of 1024 market hogs and their carcasses from four projects conducted by the National Pork Board. Live hogs were evaluated using ultrasound backfat depth and longissimus muscle area. Equations incorporating ultrasound data of live hogs and equations using carcass measures of carcass weight, 10th rib fat depth, and longissimus muscle area of carcasses had the lowest RSDs. Using ultrasound data to predict fat-free lean resulted in similar prediction accuracy as the carcass prediction equation for fat-free lean. The equation for predicting weight of fat-free lean from ultrasound data is 0.534 + 0.291 (live weight, pounds) − 16.498 (10th rib fat depth, inches) + 5.425 (10th rib longissimus muscle area, square inches) + 0.833 (1 for a barrow or 2 for a gilt). The RSD for the best equation was rather high at 3.06.

Wolf et al. (2006) evaluated the potential of incorporating ultrasound with live scores and measurements of lambs to predict percentages of carcass components and reported low R^2 and RSD values. Teixeira et al. (2006) also evaluated the potential of ultrasound technology for predicting composition of male sheep. Live weight explained most of the variation for weight of muscle and they did not report prediction equations for percentages of muscle. The correlations between ultrasound fat measures and carcass fat measures were low.

Ultrasound technology can be useful for evaluating composition of potential breeding animals, determining optimum harvest endpoints of market animals, and monitoring changes in composition as pigs grow. Prediction equations for percentages of muscle and fat are not accurate enough in sheep to be recommended for research purposes.

Specific Gravity

This technique relies on the principle that bone has a specific gravity of 1.50, muscle has a specific gravity of 1.06, and fat has a specific gravity of 0.92. This technology is more suitable for assessing fat content of carcasses than for muscle or bone because the specific gravity of a carcass does not distinguish between muscle and bone and there can be large differences in muscle to bone ratios among animals of the same species. Consequently, value differences among animals with similar fat content are not clearly distinguished. Even so, this technology is relatively accurate for determining fat differences among animals. It is a technique that can be used by institutions because there is very low financial investment and little training is required. It would not be practical for industry. And, of course, it cannot be used for live animals.

Carcass Yields of Closely Trimmed Retail Product or Fat-Free Lean

Dikeman et al. (1998) developed prediction equations from data obtained from 610 steer carcasses of different biological types to predict percentages of boneless retail product trimmed to either 0.76 or 0.00 cm fat cover using USDA yield grade traits. The equations using calculated yield grades to predict percentage of boneless retail product trimmed to 0.00 cm surface fat cover involved the parameters of *y*-intercept, calculated yield grade and (calculated yield grade)^2. The equations accounted for 58% and 57% of the variation in retail product trimmed to 0.76 cm and 0.00 cm, respectively. An equation for predicting percentages of roast and steak meat trimmed to 0.00 cm surface fat cover accounted for 90% of the variation. This likely is more meaningful economically than predicting total retail product yield. The equation for predicting *weight* of total retail product trimmed to 0.00 cm surface fat cover accounted for 86% of the variation. Interestingly, percentage of total fat could be predicted with a little higher accuracy (63%) than could percentage of retail product.

Shackelford et al. (1995) developed an equation from data from 1602 calf-fed steer carcasses from cattle of different breeds and(or) biological types to predict percentage of closely trimmed retail product of beef carcasses using wholesale rib dissected muscle, short-rib weight, and marbling score. The equation using these traits explained 87% of the variation in percentage of totally trimmed retail product. When the equation was validated against 1160 carcasses from another experiment, it explained 74% of the phenotypic variation and 96% of the genetic variation.

These authors concluded that prediction of carcass closely trimmed, boneless retail product using carcass, and wholesale rib dissection traits should allow for rapid, precise, cost-effective assessment.

This is particularly true when the objective is to determine genetic differences. In addition, these procedures do not destruct the longissimus thoracis muscle and, thus, also allow for assessment of meat palatability on the longissimus muscle after retail product data collection.

In comparing the equations of Dikeman et al. (1998) and Shackelford et al. (1995) for predicting boneless, closely trimmed retail product, the most cost-effective procedure is the equation of Dikeman et al. because the equation uses carcass traits only. The Shackelford et al. (1995) equation is more accurate, but requires dissection of the wholesale rib. The objectives of the research or industry needs should be considered when choosing which method to use.

Higbie et al. (2002) developed a prediction equation for weight of fat-free lean of pork carcasses from physical dissection of the ham. The R^2 value was 0.91 and was as good as their equation involving TOBEC and carcass weight. Although this technique is rather labor intensive and reduces the value of the ham, the fat-free lean that is dissected can still be used to make a ground ham product and much of the value can be recuperated. It certainly could be used in lieu of a TOBEC instrument when one is not accessible, and the initial cost would be dramatically less than purchasing a TOBEC instrument.

Johnson et al. (2004) published results of an extensive study of 1024 market hogs and their carcasses from four projects conducted by the National Pork Board. Carcasses were evaluated by five instrumental procedures for fat thickness and longissimus muscle depth as well as carcass measurements for predicting weight of fat-free lean. An equation incorporating carcass weight, 10th rib fat depth, and longissimus muscle area of carcasses had the lowest RSD of 2.93. However, this RSD value exceeds the commonly accepted maximum RSD of 2.5 in Europe and the United States for predicting pork carcass composition.

The National Pork Board (2000) published the following equation for predicting pounds of fat-free lean from the research of Wagner et al. (1999): 8.588 + 3.005 (longissimus muscle area) + 0.465 (hot carcass weight) – 21.896 (10th rib fat depth). Percentage of fat-free lean is determined by dividing weight of fat-free lean by hot carcass weight. For both industrial and institutional research, this equation will be quite adequate for detecting treatment, value, and genetic differences.

Summary

Livestock breeders, researchers, and meat-processing companies have a variety of options for measuring or predicting animal or carcass composition. Each must determine the objectives of determining composition and assess the availability, cost, and accuracy of the different techniques. Developments and improvements of several technologies have been made in recent years, whereas some techniques have not been proven accurate or practical enough for their continued use. Some highly accurate technologies are not accessible to most livestock breeders, researchers, and meat-processing companies because of high costs and high level of training required for their use. Users should select the method that "optimizes" accuracy, cost, availability, and objective of their use.

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3 Muscle Structure and Cytoskeletal Proteins

Chris R. Kerth

Introduction

When discussing the structure of muscle, we must first define what is meant by muscle. In the body, we find different types of muscle. Smooth, cardiac, and skeletal muscle: all have similar function as they all convert chemical energy into mechanical energy or contraction. Smooth muscles line the gastrointestinal tract and function to move the contents inside the tract from the mouth to the anus. While smooth muscle can be, and is, used as a food, it is found in small quantities, and its use is limited. Cardiac muscle, as the name implies, is the muscle of the heart. Its sole function is to pump blood, and while it too can be used as a food product, it is also considered a by-product of processing. Both cardiac and smooth muscle are involuntary muscles and their basic structure is somewhat different compared to skeletal muscle, which will be our sole topic of discussion.

Skeletal, or striated, muscle is the organ of movement in the body. It is connected to ligaments, tendons, and in some cases, directly to bone. Skeletal muscles' function is to provide locomotion, support, and movement of parts of the body necessary for life. All of these movements are voluntary, or are carried out only as needed, unlike smooth or cardiac muscles which are involuntary. Skeletal muscle also contains, within the tissue, veins and arteries, nervous tissue, and connective tissue in addition to the actual muscle cells themselves.

Connective Tissue

Collagen is the most abundant protein in the body and, because of its unique function of giving strength to muscle and other tissues, has a unique structure as well. All proteins are composed of amino acids arranged in a sequence that give a protein the specific traits or functions that it needs to carry out its mission in the body (Table 3.1) (Grant et al., 1967). Because collagen needs to be strong, its amino acid composition and sequence allow it to have these properties (Fratzl et al., 1997; Ottania et al., 2001).

The two most common amino acids found in collagen are also two that have very unique traits. Glycine is the simplest and smallest amino acid with a lone hydrogen as a side chain. Because of its small size, glycine is usually found in sequences where space is tight and steric hindrance needs

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Table 3.1 Five major types of collagen

Collagen type	Composition	Tissue distribution
I	Low hydroxylysine Low carbohydrate Broad fibrils	Tendons, bone, skin, ligaments, cornea, internal organs (~90% of body collagen)
II	High hydroxylysine High carbohydrate Thin fibrils	Cartilage, eye, intervertebral disk
III	High hydroxylysine Low carbohydrate High hydroxyproline	Blood vessels, skin, internal organs
IV	High hydroxylysine High carbohydrate	Basal laminae
V	High hydroxylysine High carbohydrate	Many tissues in small amounts

Source: Adapted from Rawn (1989).

to be kept to a minimum, which is indeed the case in collagen. Glycine makes up about one-third of all the amino acids found in collagen, indicating that one property of collagen will be very tight spaces around the peptide chain.

The other most common amino acid found in collagen is actually an imino acid. Proline and its analog hydroxyproline also make up about one-third of all of the amino acids in collagen (Ramachandrana et al., 1973). Proline is unique in that its side chain is composed of three carbons—that actually are connected at the alpha carbon on one end and the amino group of the acid on the other, forming an imino acid. Because of this unique structure, proline is most often found in peptide sequences where a bend or kink is needed in the protein structure as its side chain causes the peptide chain to turn. Lysine is another commonly found amino acid in collagen, and it along with proline are covalently modified to hydroxylysine and hydroxyproline, respectively. The hydroxylation of these amino acids occurs after they have been inserted into the peptide (Fig. 3.1)

Therefore, if one-third of amino acids are the very small glycine and one-third are proline, which introduces turns into peptide chains, it is easy to see that the primary structure of collagen will be composed of many very tight turns. A collagen molecule (tropocollagen) is composed of three left-handed polypeptide helices coiled around each other to form a right-handed supercoil. Because the polypeptide and supercoil are coiled in opposite directions, uncoiling of the polypeptides is resisted by the supercoil (Fig. 3.2). In the polypeptides, one helix makes a complete turn every 3.0 amino acid residues with a pitch of 0.31 nm (Olsen, 1963). This tight coil is possible because of tropocollagen's unusual primary structure with a regularly repeating sequence of amino acids in which a glycine is found at every third residue. Because the tropocollagen coil makes a complete turn every three residues, there is no room for any side chain larger than a hydrogen, and because of the close proximity, intermolecular hydrogen bonds are formed between the amide hydrogen of one chain with the carbonyl oxygen of an amino acid residue in an adjacent chain (Grover, 1965; Borg and Caulfield, 1980).

In addition to the hydrogen bonds, much of the strength of the collagen fibrils come from covalent bonds formed between and within tropocollagen triple helices. Unlike many proteins that form cross-links by cysteine residues, collagen is cross-linked by lysine side chains. These cross-linkages which

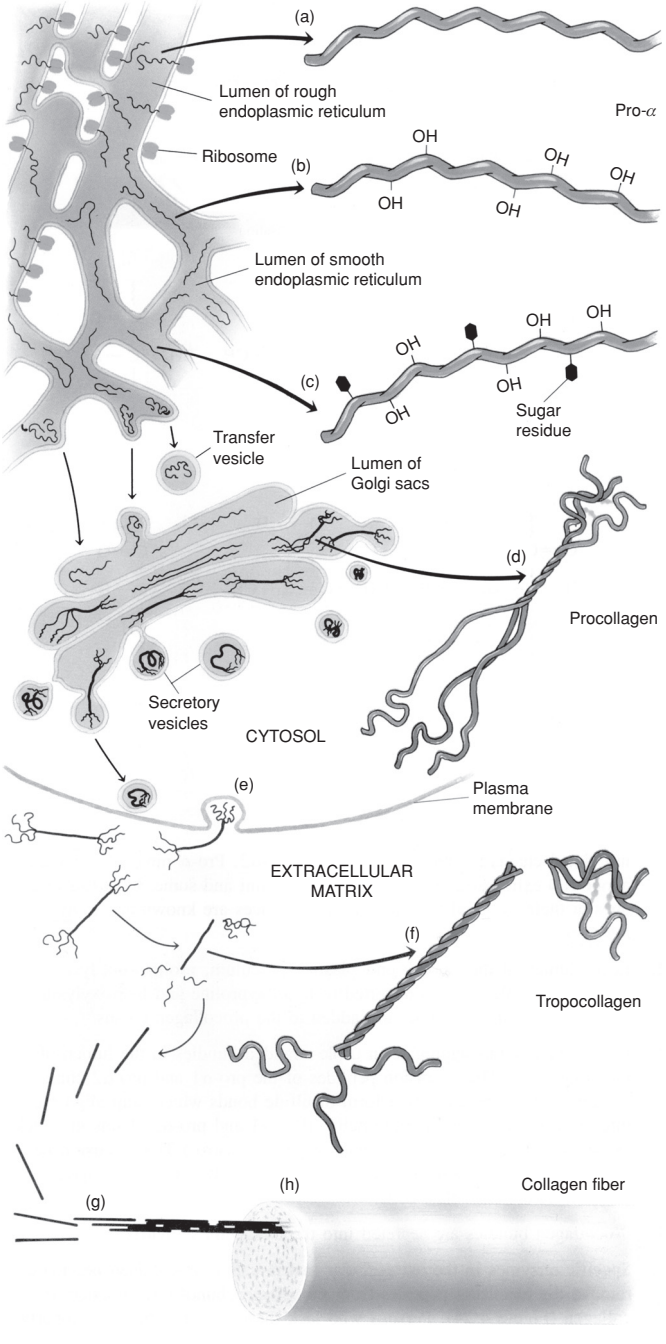

Figure 3.1 Biosynthesis and assembly of a collagen fiber: (a) Pro-alpha chains are synthesized within the rough endoplasmic reticulum; (b) proline and lysine residues are hydroxylated; (c) sugar residues are attached; (d) three pro-alpha chains assemble and form a triple alpha helix; (e) the triple helix (procollagen) is secreted from the cell; (f) extension peptides are removed from the procollagen by procollagen peptidases, tropocollagen is thus produced; (g) tropocollagen molecules aggregate and form a microfibril; (h) microfibrils aggregate and form a collagen fiber. Reproduced with permission from Rawn (1989). Copyright 1989 Carolina Biological Supply.

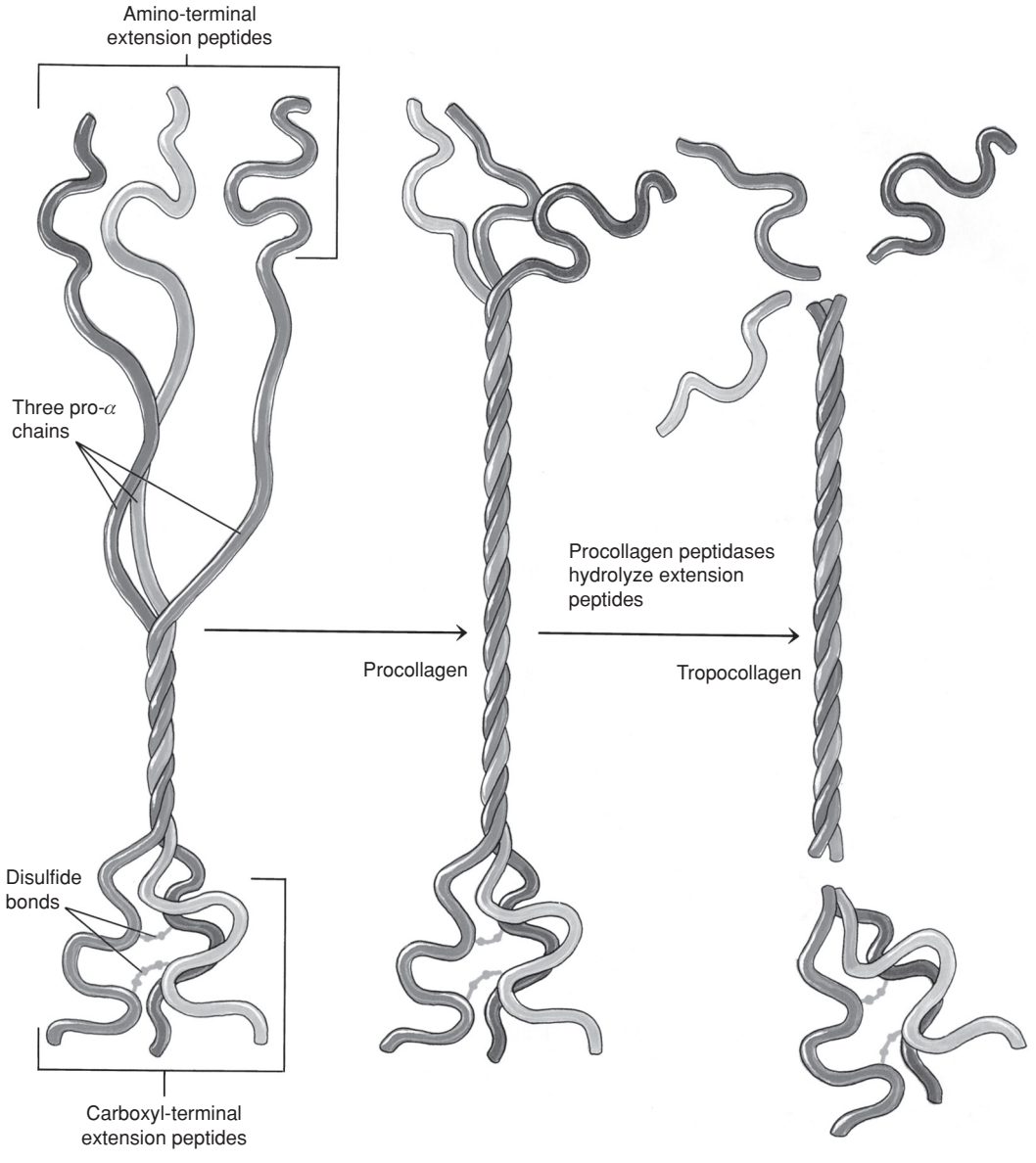

Figure 3.2 Formation of tropocollagen: extension peptides are present at the amino and carboxyl termini of pro-alpha collagen chains. Disulfide bonds between extension peptides help align the three pro-alpha chains into a triple helix. Extracellular procollagen peptidases hydrolyze extension peptides, thus producing tropocollagen. Reproduced with permission from Rawn (1989). Copyright 1989 Carolina Biological Supply.

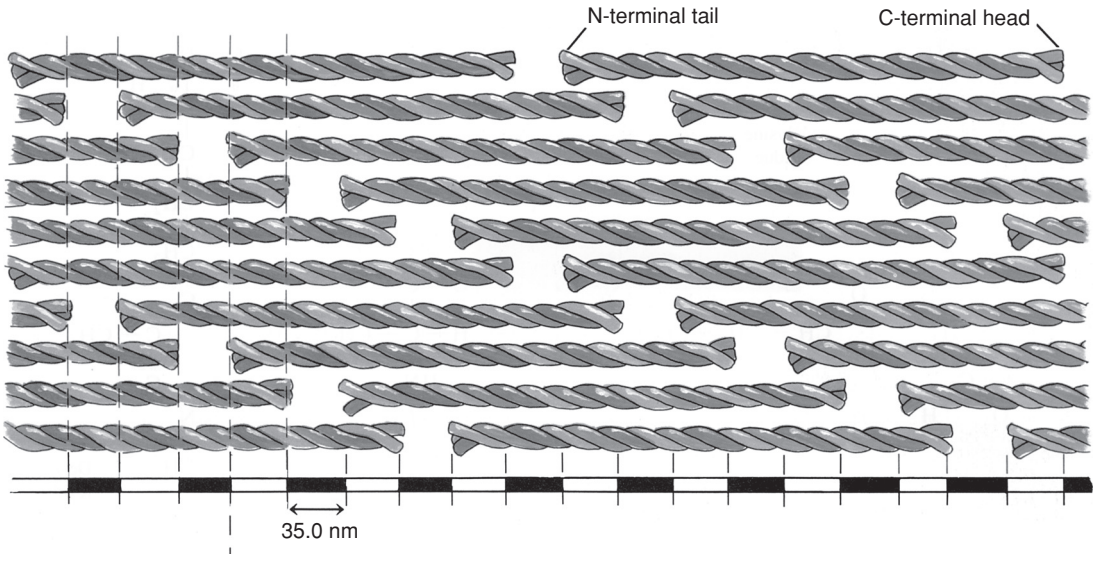

Figure 3.3 Diagram of the staggered array of tropocollagen in a microfibril: each tropocollagen molecule is displaced approximately one quarter of its length from the adjacent molecule. Deposition of heavy metal stain in the spaces at the ends of tropocollagen molecules produces the dark bands seen in electron micrographs of collagen fibers. The width of the dark band is 35 nm. A light and dark band together span 67 nm. Reproduced with permission from Rawn (1989). Copyright 1989 Carolina Biological Supply.

contribute to the strength of the collagen in meat also play a very important role in the development of meat tenderness later on (Alnaqeeb et al., 1984) (Fig. 3.3).

Organizational Structure of Muscle

Because of the complexity of its function, muscle is organized into a number of units which work together to give the muscle structure as well as its function. The whole muscle has specific origin and insertion points on the skeleton and because of its function requires a great deal of strength in its structure. A layer of collagen lays over the outside surface of the whole muscle and is called the epimysium (Rowe, 1981). The thickness of this outer layer is largely muscle-dependent and is largely correlated to the function of each individual muscle (e.g., locomotion vs. support). From an eating-quality standpoint, if the thickness of this outer layer of collagen is excessively thick, it will be trimmed from meat prior to consumption.

Within each muscle are bundles of muscle cells. Visually, these muscle bundles give the cut surface of meat its textural properties as these bundles are the smallest muscle subunit visible to the naked eye. The muscle bundles are also surrounded by a thin layer of collagen called the perimysium (Rowe, 1981). Like the epimysium, the thickness of the perimysium is dependent on muscle location and ultimately muscle function. The perimysium can have a large effect on tenderness as the type and amount of perimysial connective tissue found within the muscle may make the meat tougher. As a general rule, muscles that are used for locomotion, such as those found in the thoracic and pelvic limbs, tend to have a thicker perimysium compared to those used for support, like the longissimus and psoas muscles found along the back of an animal (Parry, 1988).

The actual cell of the muscle is the muscle fiber and as with any eukaryotic cell, the muscle cell has a cell wall (sarcolemma) and all of the organelles normally found in cells like mitochondria, nuclei,

ribosomes, etc. Unlike other cells, muscle cells also have a layer of collagen surrounding each cell just outside of the sarcolemma, called the endomysium. The endomysium gives the cell strength and elasticity, and may even play a role in the structure of the myofibrils. Like the perimysium, the endomysium may play a role in meat tenderness depending on the amount and heat-solubility traits of the collagen.

Muscle Cell Structure

As mentioned earlier, a muscle cell (or muscle fiber) has all of the organelles normally found in other cells. Unlike other cells, muscle is considered colossal in size, requiring hundreds of nuclei, and it also has a very specialized organelle that allows it to carry out its mission of converting chemical energy into mechanical energy. This very specialized organelle is the myofibril. Myofibrils run the length of the muscle fiber and are grouped within the fiber much like the muscle bundles and muscle fibers are grouped inside the muscle. These myofibrils are basically extensions of the cell membrane within the muscle fiber and contain a very complex communication system that allows a single nerve impulse to be carried not only the length of the muscle fiber but also to the center of the fiber and down to individual contractile proteins. Connecting the sarcolemma to the myofibrils are T-tubules (or transverse tubules) and longitudinal tubules that run across or lengthwise to the fiber (Block et al., 1988). The T-tubules are actually a continuation of the actual sarcolemma itself, and carry messages from the sarcolemma to the sarcoplasmic reticulum (SR). The SR is where the longitudinal tubules are found along with the fenestrated collar, which lies directly over the contractile proteins. The SR binds calcium ions and is able to release the calcium and then re-bind it in milliseconds. Just like each of the other subunits of muscle had an outer covering that helped to define it, the SR serves as the outer covering for each myofibril within the muscle fiber (Fig. 3.4) (Rossi et al., 2008; Cusimano et al., 2009).

Within the myofibril are the myofibrillar proteins (Mannherz and Goody, 1976). These proteins are also referred to as the contractile proteins as they are the specific proteins responsible for muscle contraction. The basic contractile unit within the myofibril is the sarcomere. The sarcomere structure is what gives skeletal muscle its striated appearance as each of the "stripes" that we see in micrographs corresponds to a specific structure of the sarcomere. The length of the muscle fiber and corresponding myofibrils are made up of repeating sarcomere units laid side by side. A sarcomere is made up of some basic structures. The z-disk lays perpendicular to the length of the muscle fiber and defines the sarcomere: one sarcomere is defined as the distance from one z-disk to the next z-disk (Luther, 2009). When viewing the striations of skeletal muscle, the very thin lines are the z-disks. Within the sarcomere, between the z-disks are the thick filaments and the thin filaments. These myofilaments interact with each other to carry out the actual mechanical contraction, as we will see later.

While all of the components in the sarcomere are attached to each other as we will discuss later, visually (and for the sake of understanding) the thin filaments are anchored to each z-disk and protrude to the center of the sarcomere, parallel to the length of the muscle fiber. The thick filaments appear to be "floating" in the center of the sarcomere and appear as the wide, dark bands in the striations found in micrographs. The thick and thin filaments overlap up to a certain amount depending on the state of sarcomere contraction.

We will define some other areas of the sarcomere that will help us discuss what happens in the sarcomere during contraction. The A-band is the width of the thick filament and is seen as the wide, dark band. The I-band is the light-colored band in the striations and goes from the thick filament

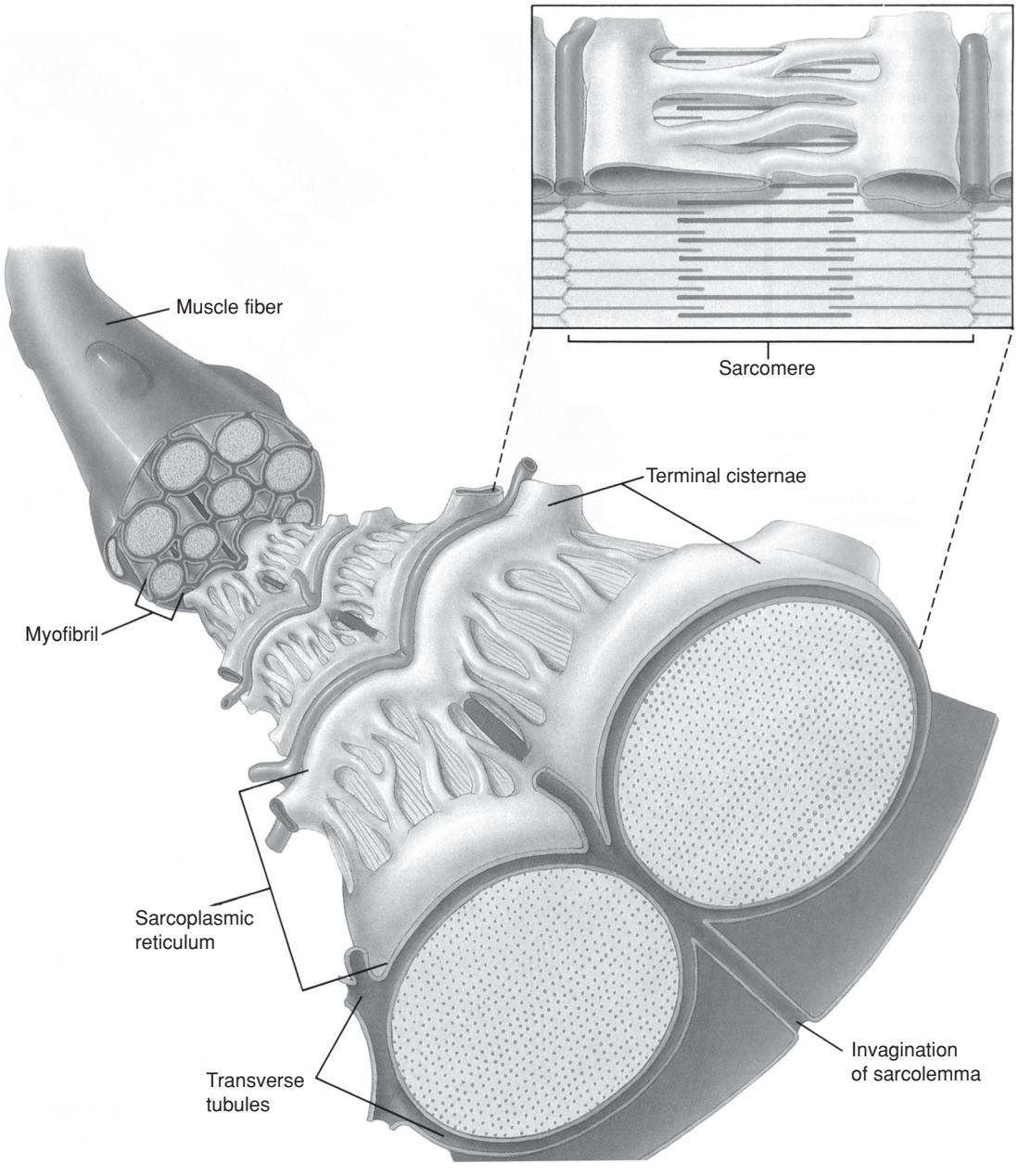

Figure 3.4 Infrastructure of a muscle fiber: muscle fibers are composed chiefly of myofibrils. Myofibrils are surrounded by a specialized endoplasmic reticulum called the sarcoplasmic reticulum, which runs parallel to the myofibrils. Another system of tubules, called transverse tubules, runs perpendicular to the myofibrils. Myofibrils are composed of smaller units called sarcomeres. Sarcomeres are composed of partially interdigitated thick and thin filaments. Reproduced with permission from Rawn (1989). Copyright 1989 Carolina Biological Supply.

or A-band in one sarcomere to the thick filament or A-band in the adjoining sarcomere. The I-band includes the z-disk found in the middle of the light-colored band. The H-zone is an area within the A-band that shows up as a lighter-colored area in the middle of this region and is the area that contains only thick filaments without any thin filament overlap. The m-line is the mid-line within the H-zone and is the mid-point of the sarcomere.

It is easy to see the different structures of the sarcomere two-dimensionally, but it is important to remember that the muscle is three-dimensional and these parts of the sarcomere occur in three dimensions. All of these structures form the tubular myofibril. Therefore, six thin filaments surround each of the thick filaments and are arranged in a very symmetrical pattern. The m-line is actually a protein that connects the adjoining thick filaments (Porzio et al., 1979; Pernigo et al., 2010), and the z-disk is a series of proteins that attach the contractile proteins in one sarcomere to the contractile proteins in the adjoining sarcomere.

Proteins of the Muscle

In addition to the discussion about the basic structure of the muscle cell and myofibrils, it is essential to understand the exact structure of the myofibril and its associated proteins if we are to understand what happens within the muscle postmortem. For discussion purposes, we will segregate these proteins into categories based on their location or function.

Contractile Proteins

As we have discussed, the thick and thin filaments in the sarcomere are responsible for doing the actual mechanical contraction. Therefore, it is essential that we understand the complete structure of these filaments to be able to understand the process of contraction.

The thick filament is made up of hundreds of molecules of the protein myosin, the most abundant myofibrillar protein in muscle. Structurally, each myosin has a long tail, a hinge, a swivel, and two heads (Fig. 3.5). Groups of myosin are bundled and held together with a cigar-band-like C-protein. To form the thick filament, the two bundles of myosin are joined tail to tail, with the tail from each bundle of myosin joining together at the m-line of the sarcomere. Remember that each thick filament is surrounded by six thin filaments, so the bundles of myosin have their heads protruding in all directions from the thick filament (Vibert and Cohen, 1988).

A molecule of myosin is actually a oligomer of a pair of identical myosin protein strands with a pair of identical heavy chains (MW = 200 kDa each) and two pairs of non-identical light chains (MW = 16 and 20 kDa each). The heavy chains have the C-terminus at the end of the tail where they form an alpha-helical rod for the tail. The N-terminus of each chain forms a globular head where each of the light chains is found. These globular heads will attach to the thin filament at the initiation of contraction. There are also two flexible regions of the myosin denoted by the swivel and hinge above. The swivel is the point at which the globular heads join the helical rods and the hinge is on the rod a short distance from the swivel. As might be expected, each of these flexible regions are susceptible to proteolysis, but it is at these regions that the myosin is able to bend, once the globular heads attach to the thin filament for contraction.

When purified myosin is incubated with trypsin (a strong protease), each chain is cleaved into two distinct fragments: a light meromyosin (LMM) and a heavy meromyosin (HMM) (Fig. 3.6).

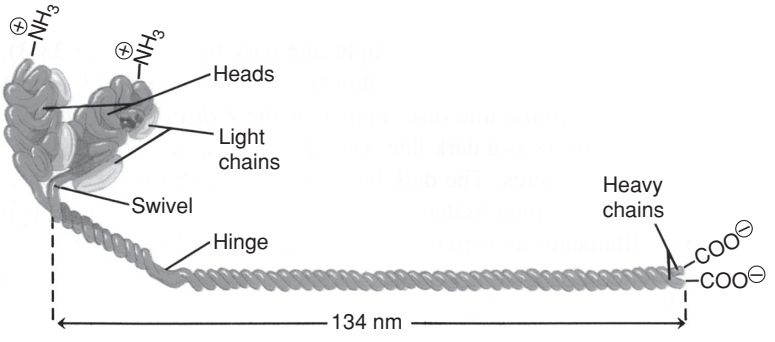

Figure 3.5 Schematic diagram of a myosin molecule: each myosin molecule is an oligomer composed of two pairs of non-identical light chains and one pair of heavy chains. The C-terminal domains of the two heavy chains are helices coiled around each other to give an alpha-helical rod. The N-terminal domain of each heavy chain forms a globular head. Each globular head is bound to one molecule of each non-identical pair of light chains. The myosin molecule contains two flexible regions. A swivel exists between the helical rod and the globular heads of the molecule. A hinge occurs in the rod itself a short distance from the swivel. Reproduced with permission from Rawn (1989). Copyright 1989 Carolina Biological Supply.

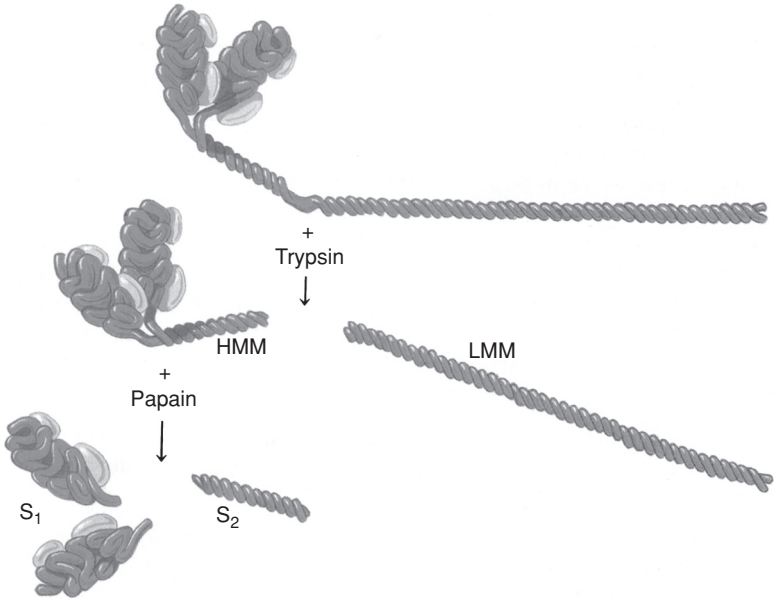

Figure 3.6 Cleavage of myosin by the proteases trypsin and papain: trypsin cleaves the myosin molecule into two fragments, called light meromyosin (LMM) and heavy meromyosin (HMM). LMM consists of most of the alpha-helical rod of the myosin molecule. HMM consists of the globular heads with the light chains and the remaining rod section. Subsequent treatment of HMM with papain releases two globular protein heads called S1 subfragments and a rod-like section called an S2 subfragment. Reproduced with permission from Rawn (1989). Copyright 1989 Carolina Biological Supply.

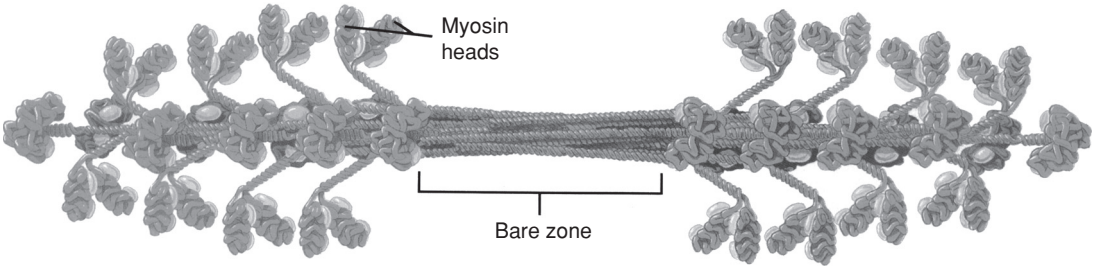

Figure 3.7 Myosin thick filament: myosin molecules associate tail to tail to form a bipolar thick filament. The myosin molecules in each half of the filament are oriented with their heads toward the nearest end, leaving a bare zone in the center of the filament. The polarity of the thick filament is reversed at the midpoint of the bare zone. Reproduced with permission from Rawn (1989). Copyright 1989 Carolina Biological Supply.

The LMM consists primarily of the helical rod section, whereas the HMM consists of the globular head along with the light chains and the remaining part of the rod. Further treatment of the HMM fragment with papain yields S1 and S2 fragments corresponding to the globular heads and the rod-like section from the hinge to the swivel, respectively. In native form, individual myosin proteins are arranged in bundles with the pairs of myosin heads protruding in all directions from the bundle. Additionally, bundles held together by the C-protein, associate tail to tail, forming a bipolar thick filament (Fig. 3.7).

Unlike the thick filament which is made up of one basic protein, the thin filament is composed of a basic structural protein along with a pair of regulatory proteins (Fig. 3.8). Actin is the basic structural protein of the thin filament. A monomeric actin molecule is a globular protein and is often referred to as G-actin. These globular monomers are then assembled into an actin filament, called F-actin (Hanson and Lowy, 1963). The length of the actin filament is regulated during assembly by two proteins: beta-actinin (Wang et al., 2010) at the end nearest the m-line (Porzio et al., 1979) and cap-Z (Pechea et al., 2007) found in the z-disk. The structure of the thin filament is composed of two actin filaments wound around themselves to form a helical strand. Like two strands of pearls wound together, the actin filaments form the thin filament with a groove forming between each strand (Kee et al., 2009).

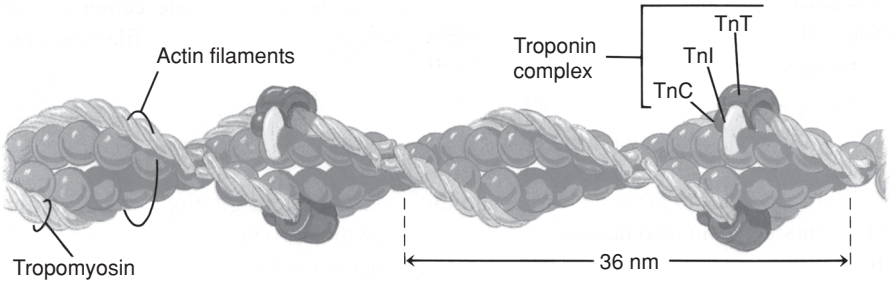

Figure 3.8 Structure of a thin filament: actin filaments form the backbone of thin filaments. These actin filaments are polymers of globular actin molecules wound around each other in a helix with a pitch of 36 nm. Tropomyosin is a helical dimer that joins head to tail to form a strand. Two strands of tropomyosin lie near the outer edges of the two grooves of the actin filament. Troponin is a trimer that binds at a specific site on each tropomyosin dimer. Reproduced with permission from Rawn (1989). Copyright 1989 Carolina Biological Supply.

Also found on the thin filament are two regulatory proteins: troponin and tropomyosin (Mika et al., 2009; Aihara et al., 2010). Tropomyosin is a long protein strand that runs the length of the thin filament, and when the muscle is at rest, lies on the outer surface of the double strand of actin. As we will see later, tropomyosin can also be shifted so that it lies in the groove formed between the two actin filaments. The function of tropomyosin is to cover the myosin binding sites on F-actin when the muscle is in a relaxed state. At the initiation of contraction, the tropomyosin is moved into the groove of F-actin exposing those binding sites.

Troponin is the other regulatory protein found on the thin filament. The troponin complex is composed of three subunits: troponin-T, troponin-I, and troponin-C, each with a very specific function. Troponin-T binds the troponin complex to tropomyosin, troponin-I binds the F-actin filament to troponin-C, and troponin-C is a calcium-binding protein, similar to calmodulin, which causes a conformational change in the troponin–tropomyosin complex resulting in the aforementioned movement of tropomyosin from the outer ridge to the groove of F-actin (Aihara et al., 2010).

Z-disk Protein

The primary z-disk protein found in the sarcomere is alpha-actinin (Mika et al., 2009). Alpha-actinin interacts with F-actin and anchors the thin filament in one sarcomere with the thin filaments of the adjoining sarcomere. Likewise, zeugmatin is found in the z-disk and interacts with both actin and titin to help anchor both filaments to the z-disk.

Gap Filaments

This group of proteins is termed "gap proteins" as they are located in the sarcomere in the "gap" between either the thick filament and the z-disk or the thin filament and the m-line. Visually, it appears that the thick and thin filaments are floating in the sarcomere with their respective ends not appearing to be connected to anything. Naturally, this is not the case. Two very important proteins are associated with the thick and thin filaments, anchoring them to the structure of the sarcomere.

Titin is one of the largest (MW = 3700 kDa) proteins found in nature and plays a crucial role in the development of the sarcomere from the time of muscle tissue development (Trinick and Tskhovrebova, 1999; Lange et al., 2005; Bertza et al., 2009). Titin spans the gap from the m-line (titin N-terminus) to the z-disk (C-terminus) of the sarcomere and actually is used as a "measuring stick" to determine the structure of the sarcomere. It has been shown that titin is one of the first proteins synthesized in myotube formation and it then acts as a scaffolding for "hanging" the rest of the myofibrillar structural proteins (Robson et al., 1997).

The part of titin that spans the A-band of the sarcomere is bound firmly to the outside of the thick filament and is relatively inelastic. The part of titin that spans from the end of the thick filament to the z-disk is very elastic, keeping the end of the thick filament in register with the z-disk and the thin filament. It is also thought that the elasticity of this portion of titin helps the extended sarcomere to return to its resting position.

Like titin, nebulin is a large (MW = 600–900 kDa) protein that helps to keep the thin filament in register (Bang et al., 2009; Chandra et al., 2009; Tonino et al., 2010). It runs parallel to the thin filament of F-actin with the N-terminus near the free end of the thin filament and the C-terminus

located in the z-disk. Nebulin's main function is to anchor the thin filament into the z-disk, but also plays a vital role in the development of the thin filament (Tonino et al., 2010). Developmentally, nebulin is found after synthesis of titin and myosin, when thin filaments of uniform length are found, therefore serving as a protein ruler for the thin filament.

Intermediate Filaments

This classification of proteins describes those proteins that, in general, run perpendicular to the length of the muscle fiber and keep the three-dimensional shape of the sarcomere and myofibrils. Their importance postmortem has become a source of much interest as it appears that these intermediate filaments and their degradation during postmortem aging may play a large part in the development of tenderness. Skelemin is found at the m-line of the sarcomere and extends to the m-line of sarcomeres in the adjoining myofibril. Desmin, paranemin, and synemin are all found at the z-disk of the sarcomere and extend to the z-disk of adjoining myofibrils (Morrison et al., 1998; Fujii et al., 2000; Hijikata et al., 2008).

Costameric Proteins

Like the intermediate filaments, the costameric filaments also function in keeping the organizational structure across the transverse portion of the muscle fiber. Unlike the intermediate filaments, however, the costameric proteins interact directly with the cell wall and possibly even with the endomysium layer outside the sarcolemma. Filamin, dystrophin, talin, and vinculin have all been identified as costameric proteins and, like the intermediate filaments, have sparked interest regarding their role in postmortem degradation and meat tenderness (Moorwood, 2008; Prins et al., 2009).

Isolating Myofibrillar Proteins

Learning about the function of the myofibrillar proteins, especially as it pertains to their disposition postmortem, requires that we be able to isolate each individual protein and quantify or observe it. The most commonly used method is sodium dodecyl sulfate (often called sodium lauryl sulfate) poly-acrylamide gel electrophoresis (SDS-PAGE). These gels, also called reducing gels, use a strong detergent (SDS) to completely denature, or unfold the proteins before loading them onto a gel apparatus. A poly-acrylamide gel utilizes acrylamide and bis-acrylamide polymers to form a matrix, that, when mixed in the appropriate ratios and concentrations, forms a gelatin-like polymer. This gel is then poured between two glass plates and affixed into a "gel rig". This rig places the gel with a small reservoir of buffer that is in contact with the top of the gel and a separate reservoir of buffer that is in contact with the bottom of the gel.

Purifying Myofibrils

Goll et al. (1974) gave a method for preparation of purified myofibrils using differential centrifugation.

Procedure

Ground or minced meat: (If you use less than 4 g of sample, use 50 mL conicals, otherwise use 50 mL centrifuge tubes.)

1. Suspend in 10 volumes (v/w) standard salt solution by homogenizing for 10 s. Depending on volume, use poly-tron or blender.
   (a) Centrifuge at 1000 *g* for 10 min.
2. Discard supernatant (contains CAF and other sarcoplasmic proteins)—be careful not to discard the "fat cap."
   (a) Re-suspend pellet in 6 volumes (v/w) of standard salt solution by homogenizing for 10 s.
   (b) Centrifuge at 1000 *g* for 10 min.
3. Discard supernatant—be careful not to discard the "fat cap."
   (a) Re-suspend pellet in 8 volumes (v/w) of standard salt solution by homogenizing for 10 s.
   (b) Pass suspension through nylon net strainer. (Tupperware, Orlando, FL, USA)
   (c) Centrifuge at 1000 *g* for 10 min.
4. Discard supernatant—be careful not to discard the "fat cap."
   (a) Re-suspend pellet in 8 volumes (v/w) of standard salt solution by homogenizing for 10 s.
   (b) Pass suspension through nylon net strainer.
   (c) Centrifuge at 1000 *g* for 10 min.

   Repeat Step 4 two more times.

   (To purify myofibrils after M.F.I., start at step 5)
5. Discard supernatant.
   (a) Re-suspend pellet in 6 volumes (v/w) of standard salt solution + 1% Triton X-100 by homogenizing for 10 s.
   (b) Centrifuge at 1500 *g* for 10 min.
6. Discard supernatant.
   (a) Re-suspend pellet in 6 volumes (v/w) of standard salt solution + 1% Triton X-100 by homogenizing for 10 s.
   (b) Centrifuge at 1500 *g* for 10 min.
7. Discard supernatant.
   (a) Re-suspend pellet in 8 volumes (v/w) of standard salt solution by stirring vigorously with polyethylene stirring rod.
   (b) Centrifuge at 1500 *g* for 10 min.
8. Discard supernatant.
   (a) Repeat step 7 two times, but suspend in 8 volumes (v/w) of 100 mM KCl instead of standard salt solution.
   (b) Centrifuge at 1500 *g* after each suspension.
9. Discard supernatant.
   (a) Re-suspend pellet in 8 volumes (v/w) of 100 mM KCl by homogenizing for 3 s.
   (b) Centrifuge at 1500 *g* for 10 min.
10. Discard supernatant.
    (a) Re-suspend pellet in 8 volumes (v/w) of 100 mM NaCl by homogenizing for 3 s.
    (b) Centrifuge at 1500 *g* for 10 min.
11. Discard supernatant.
    (a) Re-suspend pellet in 8–10 mL of 100 mM NaCl homogenizing for 3 s.
    (b) Prepare a slide for microscope to check for a protein.
    (c) Do a protein analysis of suspension.

Result

Purified myofibrils free of membranes are extracted (see table 3.2 below)

Myofibril purification solutions

Table 3.2 A list of chemicals and their weights needed to manufacture the myofibril purification solutions

Standard salt solution	**1 L**	**2 L**	
100 mM KCL	7.46 g	14.92 g	
20 mM K phosphate:			
KH_2PO_4	1.36 g	2.72 g	
$K_2H_2PO_4$	1.75 g	3.50 g	
2 mM $MgCl_2$ (MW = 95.21)	0.19 g	0.38 g	
2 mM EGT	0.76 g	1.52 g	
1 mM NaN_3 (pH 6.8)	0.065 g	0.13 g	
Standard salt solution + 1% Triton X-100	**500 mL**	**1 L**	**2 L**
Standard salt solution	495 mL	990 mL	1980 mL
Triton X-100 (weighed on a balance)	5 g	10 g	20 g
100 mM KCl	**1 L**	**2 L**	
KCl	7.46 g	14.92 g	
100 mM NaCl	**500 mL**	**1 L**	
NaCl	2.92 g	5.84 g	

Make 100 mL of 1 M NaN_3 and then add 1 mL/L to get 1 mM.

The protein sample, buffers, and gel are all at or near a neutral pH. As we know, when a protein is in an environment where the pH is above its iso-electric point, it will have a net negative charge. Most protein iso-electric points are below pH 7.0, so the myofibrillar proteins have a net negative charge. In the gel rig, a negative electrode is connected to the buffer in the top reservoir and a positive electrode is connected to the buffer in the bottom reservoir. The only thing connecting the two reservoirs of buffer that conducts electricity is the gel itself. If we place our purified myofibrillar proteins in wells at the top of the gel and then turn on the power, the current will flow from top (negative) through the gel to the bottom (positive) and the proteins, with a net negative charge, will be pulled down through the gel.

The concentration of acrylamide and the acrylamide to bis-acrylamide ratio decides the "tightness" of the acrylamide matrix. This matrix is a network of polymer strands suspended in an aqueous solution, through which the proteins must be pulled. The smaller the proteins, the easier it is for them to pass through; the larger the protein, the more difficult to pass. So, as the proteins are pulled through this matrix, they become separated from each other based on their ability to move through the acrylamide. Since all of the same proteins with the same size will move at the same rate, the same proteins end up in the same place on the gel when the power is shut off and, when stained, show up as a band on the gel. If we also put protein standards with known molecular weights on the gel, then we can determine the molecular weight of various bands and extrapolate to the known myofibrillar protein as shown in Table 3.3.

In setting up a gel, it is important to know what proteins you want to isolate and the approximate size of those proteins. Today, most gels may be purchased pre-made with any combination of traits needed. The most commonly used criteria for selecting the right gel is the percent acrylamide. The percentages range from about 4% to 20%. Naturally, a 4% gel would be used to isolate very large proteins, whereas a 20% gel would only be used for very small proteins. Often, a whole range of proteins are of interest, like the myofibrillar proteins, and a gradient gel is very useful. A 4–12%

Table 3.3 Common structural proteins found in skeletal muscle

Protein	Approximate MW (kDa)	Location/function
Titin	3700	Thick filament/gap
Dystrophin	854	Costameric
Nebulin	773	Thin filament/gap
Filamin	560	z-disk peripheral/costameric
Talin	536	Costameric
Myosin	520	Thick filament/contraction
Synemin	372	z-disk peripheral/intermediate
Paranemin	356	z-disk peripheral/intermediate
Desmin	212	z-disk peripheral/intermediate
Alpha-actinin	204	z-disk/anchors thin and thick
Skelemin	195	m-line intermediate
Myomesin	185	Keeps myosin in register at m-line
M-protein	165	Keeps myosin in register at m-line
C-protein	130	Bundles myosin in thick filament
Vinculin	116	Costameric
Troponin	69	Thin filament/regulatory
Tropomyosin	66	Thin filament/regulatory
Cap-z	66	z-disk/regulates thin filament size
Actin	42	Thin filament/structural

or 4–15% gradient gel is useful for isolating all of the myofibrillar proteins from the largest to the smallest. It is worth noting that to isolate more proteins it requires larger gel to get good separation.

A continuation of this technique is necessary when a specific protein needs to not only be isolated but also its specific identity, along with any of the parts of this protein lost to degradation, be identified. To accomplish this, we would conduct a Western blot analysis.

We begin by running our proteins out on a gel as we have described. We then make a "sandwich" out of the gel and a blotting paper. Then, using the same principle of negative to positive electrical current as the gel, we transfer the proteins sideways onto the blotting paper. Then, using antibodies specific to our protein of interest, we can stain and identify the protein and any of its degradation products.

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4 Muscle Metabolism and Contraction

Chris R. Kerth

Introduction

While we are concerned with quality of meat as a food, it is vitally important that we understand the basic functions of metabolism as it pertains to the muscle. The use of chemical energy in the form of adenosine tri-phosphate (ATP) to convert to mechanical energy (contraction) is the basic function of muscle in the live animal. As we will discuss in later chapters, the process of rigor centers around the process of contraction and most importantly what happens when the muscle no longer has ATP for muscle function. In this chapter we will review the basic concepts of the generation of ATP in the muscle and the unique way in which this influences muscle tissue. We will also discuss, in detail, the steps in muscle contraction, paying close attention to those steps that require ATP.

Metabolism

The concept of metabolism in the muscle is much like metabolism in other tissues in the body. Substrates like sugars, proteins, or fats are transported to a tissue cell, across the cell wall and then utilized within the cell to convert to ATP, the basic energy source for carrying out basic cellular functions. As we know, basic metabolism falls into one of the two very basic types: glycolysis or oxidative phosphorylation (the TCA or Kreb's cycle). We will briefly review each of these processes, but further details of the evolution of metabolic pathways can be found in Baldwin and Krebs (1981).

Glycolysis

The generation of ATP from sugar is done through the glycolytic pathway (see Fothergill-Gilmore and Michels, 1993 for an in-depth review). Muscle cells have the ability to stockpile a small amount of glucose in the form of glycogen. Unlike other types of metabolism, glycolysis takes place in the extracellular fluid or sarcoplasm where glycolytic enzymes are present to take the substrate through each step. The initial steps of glycolysis converts one 6-carbon molecule of glucose into two 3-carbon molecules of glyceraldehyde 3-phosphate (G3P). This requires the utilization of two

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ATP. The final steps convert each G3P to pyruvate, yielding two ATP for each G3P. Therefore, glycolysis alone generates a total of four ATP and uses two ATP resulting in a net production of two ATP.

During the final step of glycolysis, the environment in the muscle determines the next step. Under aerobic conditions in the presence of oxygen, pyruvate is converted to acetyl coenzyme (acetyl CoA) and can enter the citric acid cycle to generate two additional ATP from substrate-level phosphorylation. Under anaerobic conditions in the absence of oxygen, pyruvate is converted to lactic acid which cannot be utilized in the muscle. At this point, lactic acid is transported out of the muscle cell, enters the blood stream, and is carried to the liver where it is reconverted to glucose. The glucose is then sent back into the blood stream, transported to the muscle cell where it must then re-enter glycolysis to be metabolized to pyruvate again.

Using glycolysis as a system to produce ATP in muscle has both advantages and disadvantages. Because relatively few steps are involved, the ATP can be generated relatively quickly as long as a substrate, glucose, is available. Glycolysis can generate ATP under either aerobic or anaerobic conditions making it very useful when the muscle is not receiving sufficient oxygen. On the other hand, glycolysis is pretty inefficient, generating only a net of two ATP for each molecule of glucose under anaerobic conditions. This inefficiency is further magnified under anaerobic conditions when additional energy is required to send the lactate back to the liver to convert it back to glucose.

Oxidative Phosphorylation

The citric acid cycle is the central pathway for aerobic metabolism and is the final oxidative pathway in the catabolism of carbohydrates, fatty acids, and amino acids (Krebs, 1970). Once pyruvate is generated from glycolysis, it can enter the mitochondrial matrix to be converted to acetyl CoA. It then goes through eight enzyme-catalyzed, citric acid cycle reactions in the mitochondria producing three NADH and one $FADH_2$. It is here that two ATP can be generated alone through substrate-level phosphorylation. The NADH and $FADH_2$ proceed into the electron transport chain where 34 ATP are generated by oxidative phosphorylation. Certainly, the efficiency of ATP production under aerobic conditions is vastly superior to that of glycolysis, particularly when glycolysis is operating under anaerobic conditions. The only real disadvantage is that this system relies heavily on the presence of oxygen to be able to carry out its functions. If conditions exist where oxygen becomes limiting, then the only method of ATP production is through anaerobic glycolysis.

Therefore, under aerobic conditions, one molecule of glucose can generate a net total of 38 ATP: 2 from glycolysis, 2 from substrate-level phosphorylation in the citric acid cycle, and 34 from oxidative phosphorylation in the electron transport chain. Certainly, the environment inside the muscle plays a major role in the ability of that muscle to be able to contract. This fact becomes essential to understand the process of rigor discussed in the next chapter.

Muscle Contraction

To understand the process of rigor in the next chapter, it is essential that we have a thorough understanding of the process of muscle contraction. For this process, we will discuss the formation of a nerve impulse, the transfer of that nerve impulse to the muscle cell or fiber, the mechanisms of converting chemical energy into mechanical energy, and finally muscle relaxation.

Nerve Impulse

A history of the nerve impulse can be found in the work of Bishop (1956). The basic structure of a nerve starts with the cell body. From this cell body, numerous dendrites extend and serve as receptors of signals from other cells. These signals are then transmitted from the cell body along the nerve axon to axon terminals which contact the target tissue or next neuron. The plasma membrane of the axon has a membrane potential. This is possible because of the maintenance of sodium (Na^+) and potassium (K^+) gradients formed across the axon plasma membrane (Costantin, 1970). This gradient is maintained by a sodium/potassium pump or sodium/potassium ATPase which is required to move each of the respective positive ions across the plasma membrane against a concentration gradient. As a result, there is a high concentration of sodium outside the membrane and a high concentration of potassium inside the membrane. This results in the interior of the plasma membrane of the nerve axon having a slightly negative charge (-70 mV).

When a signal is received by the cell body, it is then transported along the axon to the target tissue. This is accomplished by reversing the polarity of the membrane potential of the axon plasma membrane. A flow of ions passes over the plasma membrane causing a depolarization of the resting membrane. This initial depolarization (to about -50 mV) induces the opening of adjacent voltage-gated sodium channels in the plasma membrane allowing the sodium ions on the external surface of the membrane to flood into the axon. As the sodium ions flow into the axon, the membrane potential reverses polarity briefly, and briefly spikes to about $+40$ mV, causing a 110 mV change from its resting state. This reverse in polarity then induces the opening of sodium channels down the axon.

The sodium channels remain open for about 1 ms allowing about 6000 sodium ions to flow into the axon with its concentration gradient. At the same time, potassium channels are also opened, but they are opened more slowly and for a shorter duration than the sodium channels. The sodium/potassium pump—which requires the hydrolysis of an ATP by its ATPase—then pumps the sodium and potassium against their respective concentration gradients to the exterior and interior of the plasma membrane, respectively. The end result is a voltage spike inside of the nerve axon that is able to travel the length of the axon to the axon terminal (Adrian and Bronk, 1929).

At the end of the nerve axon is the axon terminus that is attached to the target tissue, in this case muscle, to form a contact site, called a synapse. A presynaptic membrane is found on the nerve terminus, followed by a space called the synaptic cleft, followed by the postsynaptic membrane on the muscle. When the nerve impulse arrives at the axon terminal, an additional ion channel is then opened in a method similar to the voltage-gated channels used for sodium and potassium. In this case, they are voltage-gated calcium channels. The sudden depolarization of the axonal terminal membrane causes an influx of calcium ions into the cytosol of the axon. This causes synaptic vesicles to fuse with the synaptic membrane, releasing the vesicle contents, acetylcholine (Krnjevic and Miledi, 1958; Drachman, 1974). Acetylcholine is synthesized by transfer of the acetyl group from acetyl CoA to choline and is used as a neurotransmitter from the motor neurons to the muscle cell. The acetylcholine diffuses across the 50 nm synaptic cleft to the acetylcholine receptors of the muscle cell. The binding of acetylcholine on the postsynaptic membrane of the muscle fiber causes a conformational shift in the receptor protein which allows an influx of cations, particularly sodium ions. This influx then causes a depolarization of the muscle cell membrane, the sarcolemma, just as we saw in the nerve axon from about -75 to 0 mV. As we saw in the previous chapter, the sarcolemma membrane is extended to the interior of the muscle cell through the transverse tubules, so when the sarcolemma is depolarized, the signal is transmitted throughout the entire muscle cell through this vast intracellular communication system (Katz and Thesleff, 1957; Howell, 1969) (Fig. 4.1).

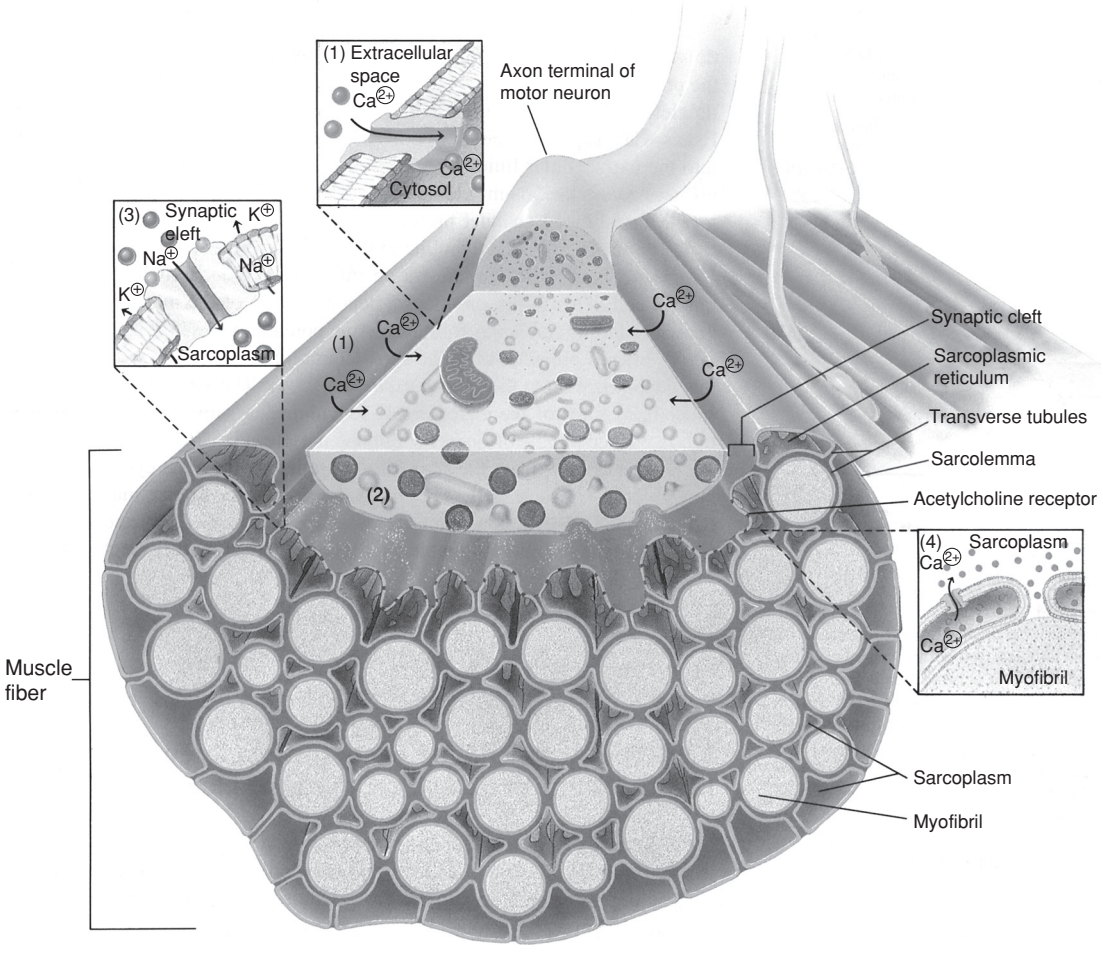

Figure 4.1 Signal transduction at a neuromuscular junction. When an electrical signal reaches the presynaptic membrane of a resting neuromuscular junction, the following events ensue. (1) Depolarization of the axonal membrane causes a voltage-gated Ca^{2+} channel to open. (2) The influx of calcium into the cytosol of the axon terminal stimulates the fusion of synaptic vesicles with the presynaptic membrane and the release of acetylcholine into the synaptic cleft. (3) Acetylcholine diffuses across the synaptic cleft to the postsynaptic membrane, where it binds to a ligand-gated channel protein called an acetylcholine receptor. Binding of acetylcholine causes the cation channel to open, and the subsequent influx of cations, primarily sodium, results in depolarization of the sarcolemma of the muscle cell. This depolarization is transmitted via the transverse tubules to the sarcoplasmic reticulum, which surrounds each myofibril. (4) Voltage-gated calcium channels in the sarcoplasmic reticulum open, permitting calcium ions to flow from the lumen of the sarcoplasmic reticulum into the sarcoplasm of the muscle cell. Reproduced with permission from Rawn, 1989. Copyright 1989 Carolina Biological Supply Company.

The impulse travels along the transverse tubule until it reaches the junction between the tubule and the sarcoplasmic reticulum called the triad (Block et al., 1988; Ito et al., 2001). At this junction are two specific proteins that are responsible for translating a membrane potential into a release of calcium (Marty et al., 1994). The first is the dihydropyridine receptor which is a voltage-gated calcium channel in the T-tubule and the other is the ryanodine receptor (Fill and Copello, 2002; Lanner et al., 2010), which is a calcium release channel found in the sarcoplasmic reticulum.

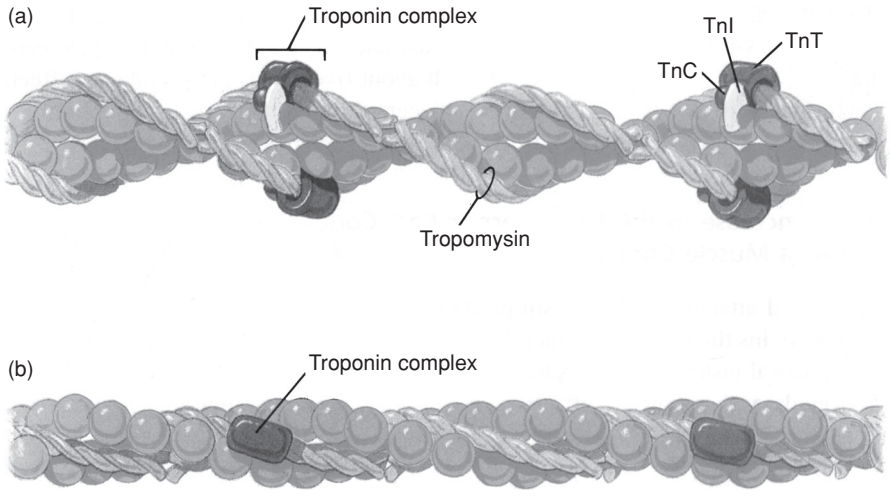

Figure 4.2 Change in conformation of a thin filament during muscle contraction. (a) Thin filament in the absence of calcium ions. (b) Thin filament in the presence of calcium ions. It is believed that the binding of calcium ions to the troponin C subunit of troponin triggers the movement of the troponin–tropomyosin complex deeper between the two strands of the actin filament, which stimulates the ATPase activity of myosin. Reproduced with permission from Rawn, 1989. Copyright 1989 Carolina Biological Supply Company.

Therefore, the T-tubule depolarization causes a conformational change in the calcium channel allowing calcium to be released into the sarcoplasm (Flucher, 1992). Two additional proteins of importance in the sarcoplasmic reticulum (SR) are SR calcium ATPase (Barany, 1967) and calsequestrin (Paolini et al., 2007). The SR calcium ATPase is a calcium pump that is able to transport two calcium ions from the sarcoplasm to the SR with the hydrolysis of an ATP and pumps calcium into the SR against a concentration gradient. Calsequestrin is a low-affinity, high-capacity calcium binding protein with 40–60 calcium binding sites that functions as a calcium buffer able to maintain calcium concentrations in the mM range within the SR (Treves et al., 2009).

Once the release of calcium from the SR is triggered, the sarcoplasmic concentration of calcium ions causes a 100-fold increase in calcium from 10^{-7} to 10^{-5} M (Endo, 1977). With this sharp increase in calcium concentration, calcium binds to troponin C in the troponin complex (Herzberg et al., 1986). This causes a conformational shift in the troponin complex, and since troponin is directly connected to tropomyosin, also shifts the tropomyosin from the outer ridge of the thin filament, and into the groove formed between the two F-actin strands (Farah and Reinach, 1995; Shitaka et al., 2004). The myosin binding sites found on the actin filament are found on the outer ridge, so when tropomyosin slides down into the groove, these binding sites become uncovered and available for the myosin heads to bind (Parry, 1973; Zot and Potter, 1987) (Fig. 4.2).

As previously discussed, the myosin head or S1 heavy meromyosin subunit contains ATPase along with an actin binding site (Vibert and Cohen, 1988; Heeley et al., 2006). In its resting state, the myosin head has an ADP and inorganic phosphate (Pi) bound to it. Once the binding sites are uncovered on the thin filament, the myosin head will bind to the actin filament to form an actomyosin complex (Walker et al., 2000). Once this complex is formed, the ADP + Pi is released from the myosin head causing a conformational shift at the hinge of the myosin heavy chain (MHC). This conformational shift comes in the form of a head "tilt" toward the tail of the myosin, or toward the m-line of the sarcomere. This is called the "power stroke." A new ATP then binds to the myosin

head detaching the myosin head from the thin filament and dissociating the actomyosin complex. The ATP is then hydrolyzed to ADP + Pi by the ATPase on the myosin head causing the myosin head to tilt back to its original position. This cycle continues as long as the binding sites are uncovered on the thin filament causing an incremental shortening of the sarcomere. Each power stroke of the myosin head results in moving the thin filament 4–5 nm closer to the m-line. Another mechanism has recently been proposed that suggests that myosin V and VI (molecular motors) can walk progressively along the actin filament with a 36 nm step (Walker et al., 2000; Okten et al., 2004). Regardless of the specific mechanism, the end result is a ratcheting effect that can ultimately result in the shortening of a sarcomere from about 2.7 μm to about 1.5 μm. It is also important to remember that each thick filament is surrounded by six thin filaments, so the ratcheting mechanism is taking place in the three-dimensional space surrounding each thick filament. Furthermore, since sarcomeres are repeating units found in the length of the muscle fiber, the 1 μm shortening found in one sarcomere is multiplied by the thousands of repeating sarcomeres resulting in a whole muscle fiber able to contract a total of several centimeters in length (see also Rayment et al., 1993; Geeves and Holmes, 1999) (Fig. 4.3).

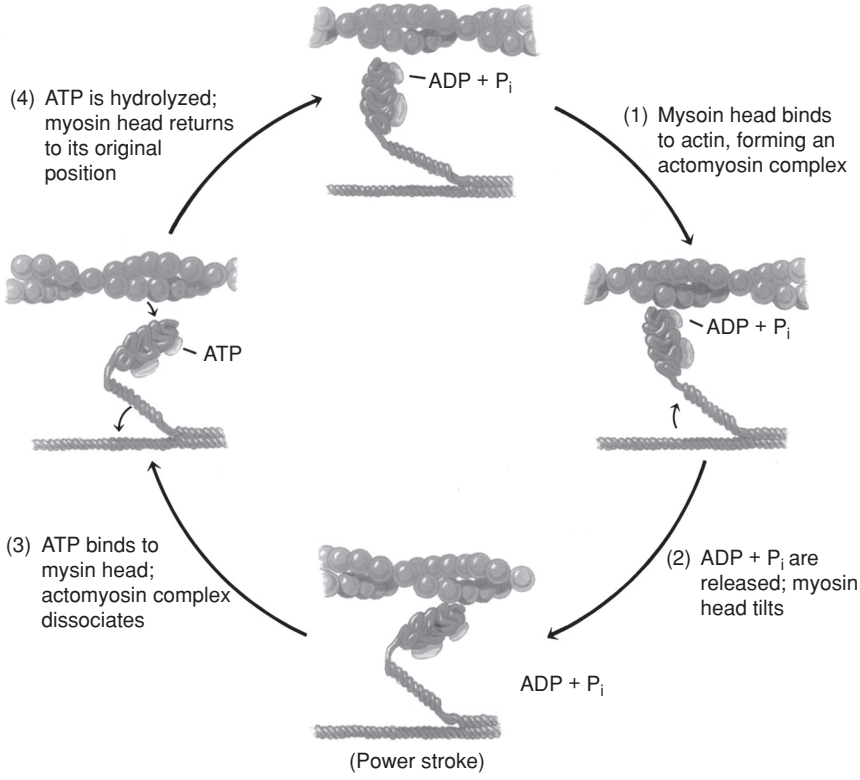

Figure 4.3 Proposed mechanism for muscle contraction in which energy recovered from ATP hydrolysis triggers a conformational change in myosin heads. (1) A myosin head binds to the actin backbone of a thin filament, forming an actomyosin complex. (2) Myosin releases ADP and Pi and undergoes a conformational change. The change in myosin conformation provides the power stroke of muscle contraction. (3) ATP binds to the myosin head, causing the head to dissociate from actin. (4) ATP is hydrolyzed to ADP and Pi, and the head returns to its original position. (Note that only the actin backbone of the thin filament is shown. Note also that the second myosin head has been deleted for clarity.) Reproduced with permission from Rawn, 1989. Copyright 1989 Carolina Biological Supply Company.

Relaxation

While muscle relaxation is more or less contraction in reverse, it is important to go through the steps in relaxation making particular note of the steps that require ATP as those steps become limiting when we discuss the process of rigor. Again starting with a nerve impulse, the absence or removal of a stimulus to the cell body results in returning the membrane potential to its resting state with concentration gradients of sodium and potassium and a slightly negative charge in the interior of the axon. In the absence of depolarization of the terminal membrane of the axon, acetylcholine is no longer released and cholinesterase very quickly breaks down acetylcholine present in the synaptic cleft. Without acetylcholine, the receptors in the postsynaptic membrane close and the influx of cations into the sarcolemma ceases with those ions being actively pumped into the extracellular space. The action potential ceases to flow through the T-tubules and the dihydropyridine and ryanodine receptors return to their resting state. Calcium is actively pumped out of the sarcoplasm and into the SR by SR calcium ATPase, reducing sarcoplasmic calcium concentrations to resting levels (Ostwald and MacLennan, 1974). Calcium dissociates from the troponin complex causing the conformational shift in troponin and the resulting movement of tropomyosin out of the actin groove and back over the myosin binding sites. Finally, as ATP is bound by myosin to dissociate actomyosin complex (Holmes et al., 2003), the binding sites are covered and myosin is not able to bind to the thin filament. Passive sliding of the thin filaments will take place until contraction begins again.

Muscle Fiber Types

We have discussed, in detail, the basic structure of a muscle fiber, but the fact is that there are many different types of muscle fibers. The type of muscle fiber is determined based on two very basic principles: contraction speed (related to myosin isoform) and type of metabolism. Postnatal skeletal muscle is known to contain four different isoforms of the MHC. These MHC isoforms are directly related to their contraction speed increasing from Types Ia, IIa, IIx, to IIb (Bottinelli et al., 1991; Larsson and Moss, 1993). In general terms, Type I are considered slow-twitch and Type II fast-twitch. The speed of contraction is also related to the specific ATPase activity found on each MHC. Type II fiber types are further divided into Types IIa, IIx, and IIb based on the predominant type of metabolism found within each respective fiber. Types IIa and IIx are often lumped together and categorized as “intermediate” fiber types, but Type IIa relies very heavily on oxidative metabolism for generating ATP whereas Type IIx is much more glycolytic in nature.

Because the process of contraction requires one ATP for each attachment, powerstroke, and release of the myosin head, the speed at which the ATP can by hydrolyzed by the ATPase activity of the myosin head determines the ultimate speed at which contraction can take place. Slow, methodical, or tonic muscle contractions are found in muscles with a high proportion of Type I fibers. In contrast, rapid, phasic contractions are the result of a high proportion of Type II fiber types.

We can further describe the different types of muscle fibers based on the type of metabolism mostly used in the fiber (Peter et al., 1972). Types I and IIa fibers rely very heavily on oxidative metabolism, meaning that these fibers generate ATP almost exclusively in the mitochondria from the citric acid cycle and electron transport chain. As a result, the histology of these types of fibers includes all of the organelles and structures that would be required to carry out this type of metabolism. Because oxidative metabolism is carried out solely in the mitochondria, these fiber types have many large mitochondria in the cells. In addition, since oxidative metabolism can occur only in an aerobic

environment, it is essential that an adequate oxygen supply be present in these types of fibers. As a result, Type I and IIa fibers contain a high concentration of myoglobin which is the carrier of oxygen and carbon dioxide within the muscle and is the analog to hemoglobin found in the blood stream. These types of fibers also will be highly vascularized as a large number of veins, arteries, and their associated capillaries are necessary to carry the oxygen from the lungs to the muscle fibers. These fibers also tend to be rather small compared to the other two fiber types and tend to have relatively thin z-disc.

To fully visualize the function of Type I and IIa fibers, it is helpful to imagine the type of work that muscles with these types of fibers will do. In humans, we can imagine a marathon runner who runs more than 26 miles without stopping for rest. This runner does not run quickly, but a methodical, steady rhythm is necessary to be able to continue this constant state of contraction and relaxation. The gross muscle anatomy of these runners tends to be very thin with relatively small, but defined muscles. In animals, we think of migratory birds who will fly maybe 100 miles in a day. The pectoral muscles required to flap their wings must continue to contract and relax all day without stopping. Full development of strong heart and lungs is vital in these two examples in order to continue supplying an adequate supply of oxygen to these oxidative muscles. In addition, the substrate that is used for oxidative fibers, fat in particular, is in much higher levels in the oxidative type muscle fibers.

In the much more glycolytic Type IIx and IIb muscle fibers, we find them to be much larger in diameter and lacking the extensive vascularization, myoglobin content, and mitochondria found in the other two fiber types. These two fiber types rely very heavily on glycolytic metabolism which takes place in the free cellular space or sarcoplasm. If we remember, glycolysis can operate in either aerobic or anaerobic conditions, but is very inefficient in the production of ATP. Again, if we imagine these muscle fiber types at work, in humans we think of a 100 m sprinter or weight lifter. These individuals have very quick and powerful muscle contractions, but the endurance of these muscles is extremely limited. The muscle physiology is characterized by very large, bulging muscles. When we exercise using very rapid contractions as in weight lifting, or even on a treadmill, if we begin exerting our muscles beyond their capabilities, we quickly begin to feel them "burn." This is because the muscle is not getting enough oxygen to them for oxidative metabolism and glycolysis is not able to keep up with ATP production to maintain the contraction and relaxation of the muscle, so glycolysis begins to produce ATP anaerobically, which ultimately produces lactic acid. The lactic acid is what we feel as the "burn." That is why many times training coaches tell their athletes to "feel the burn," meaning that they want the athletes to push their muscles to exertion for training purposes. As we saw in the oxidative fibers, the glycolytic fibers rely on glucose as the substrate for metabolism, so the amount of stored glucose (glycogen) in these muscles is particularly high (Fig. 4.4).

Because myosin is a protein, each muscle is created with a genetic predisposition to have a certain mix of the four myosin isoforms along with their respective metabolic enzymes and organelles necessary to carry out their function. We also know that as we make genetic selections for certain muscle traits, we will affect the composition of the relative muscle fiber types. Selection for muscle growth will result in the selection for a higher proportion of Type IIb muscle fiber types. In poultry, the broiler or turkey are prime examples of this. Over generations of selection for the growth of breast meat, the pectoralis muscle is now almost 100% Type IIb muscle fibers. In fact, the reason that we find "white meat" and "dark meat" in poultry is because of the difference in distribution of muscle fiber types. Muscles that are still used by the animal for locomotion (muscles of the leg and thigh) still have a relatively high proportion of oxidative type muscle fibers that have a high

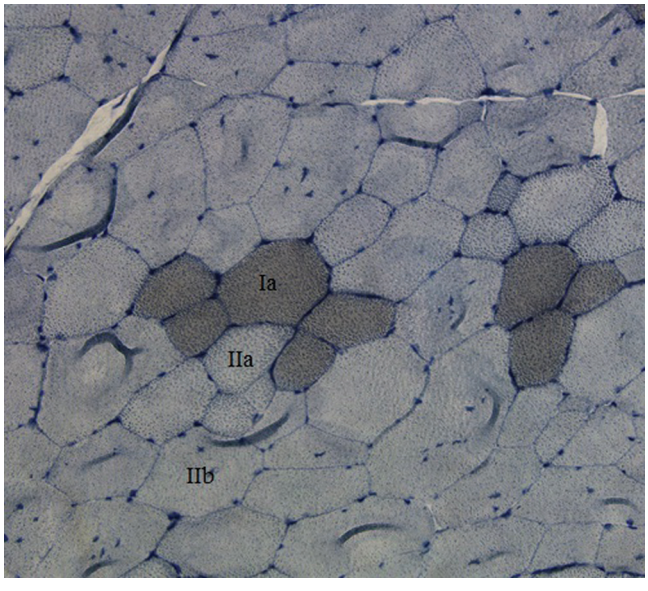

Figure 4.4 Cross-section of pork muscle 40 × magnification. Type Ia (red), Type IIa (intermediate), and Type IIb (white) fiber types are shown. The dark spots around the periphery of the fiber indicate cell nuclei and the small dark spots, or "grains" within the fiber mitochondria. Note that Types Ia and IIa have many more mitochondria compared to Type IIb.

amount of myoglobin, while the breast meat remains white in the absence of myoglobin. In wild fowl where natural selection still requires heavy use of flight, the breast muscle is very dark in color because the oxidative muscle fiber types are still found in high quantities compared to domesticated birds.

Fiber Typing Procedure—Combined Stain for Identifying Muscle Fiber Types

Principle

Unfixed tissue sections are stained first for metabolic function of NADH and then for physiological function by the acid–ATPase reaction.

Solutions

1. Acid pre-incubation solution
   100 mL ***$CaCl_2$ (0.18 M)***
   3 mL ***Glacial acetic acid***
   890 mL ***ddH_2O***
   Adjust pH to 4.15, QS 1 L
   Note: Even though the pH is adjusted at this step, it will need to be adjusted again depending on the species being typed.

2. Rinse
   12.1 g ***Tris Base***
   100 mL ***$CaCl_2$ (0.18 M)***
   900 mL ***ddH_2O***
   Adjust pH to 7.8
3. ATPase incubation medium (make fresh immediately before use)

40 mL batch	*200 mL batch*
2.68 mL ***2-amino 2-methyl 1-propanol (1.5 M)***	13.4 mL
4 mL ***$CaCl_2$***	20 mL
0.148 g ***KCl***	0.740 g
0.0608 g ***ATP***	0.304 g
32 mL ***ddH_2O***	160 mL
(QS to 40 mL)	(QS to 200 mL)

   Adjust pH to 9.4; incubate tissues to 37°C
4. β-NADH incubation solution (*make fresh daily*)
   10 mL ***0.2 M Tris buffer***, pH 7.4
   10 mg ***tetranitro blue tetrazolium (TNBT)***
   8 mg ***nicotinamide adenine dinucleotide, reduced***
   Note: No matter how long you mix this, it never seems to go into solution. Therefore, it *must be filtered*.
5. Tris buffer, pH 7.4
   75 mL ***0.2 M Tris base (12.11 g/500 mL)***
   126 mL ***0.1 M HCl (50 mL of 1 N HCl, QS 500 mL;*** where ***1 N HCl*** is 41.7 mL HCl/458 mL ddH_20)
   174 mL ***ddH_2O***
   As a preservative, add a few drops of ***chloroform***.
6. Ehrlich's hematoxylin stain
   6 g ***hematoxylin***
   300 mL ***ethyl alcohol, absolute***
   9 g ***aluminum ammonium sulfate***
   300 mL ***ddH_2O***
   300 mL ***glycerin (glycerol)***
   0.72 g ***sodium iodate***
   30 mL ***glacial acetic acid***
   Mix & filter
   Note: The stain can be reused several times.
7. **0.18 M $CaCl_2$**
   26.46 g/L
8. **1% (w/v) $CaCl_2$**
   10 g/L
9. **2% (w/v) $CoCl_2$**
   10 g/500 mL
10. **2% (v/v) ammonium sulfide** (must mix under the hood)
    10 mL/500 mL

11. **50% (v/v) ethyl alcohol**
    250 mL in 250 mL distilled H_2O
12. **Glycerol gelatin/fluoromount-G (to mount slides)**
    The glycerol needs to be warmed before use. Keep a beaker of water on a hot plate (lowest setting) and keep the glycerol bottle in the beaker while mounting slides.

Staining Sequence for Bovine

1. β-NADH solution, 45 min at 37°C
2. Distilled water, 30 s
3. Acid pre-incubation solution, *pH 4.3*; 10 min
4. Rinse solution, pH 7.8; 1 min
5. Rinse solution, pH 7.8; 1 min
6. ATPase incubation solution, pH 9.4; 30 min at 37°C
7. 1% $CaCl_2$, 30 s
8. 1% $CaCl_2$, 30 s
9. 1% $CaCl_2$, 30 s
10. 2% $CoCl_2$, 3 min
11. Distilled water, 30 s
12. Distilled water, 30 s
13. Distilled water, 30 s
14. Distilled water, 30 s
15. 2% ammonium sulfide, 3 min *(do this step in the hood)*
16. Running distilled water, 3 min
17. Hematoxylin, 5 min
18. Running distilled water, 3 min
19. 50% EtOH, 2 min
20. Drain and mount with glycerol gel

Results

β-Red fibers: Fibers that stain dark purple
α-Red fibers: Fibers that stain intermediate
α-White fibers: Fibers that stain light purple

Note

- For "cleaner" slides, filter the NADH solution before using it.
- If three fiber types are not identifiable, increase the pH of the acid pre-incubation solution until desired intensity is obtained.

Staining Sequence for Ovine

1. Acid pre-incubation solution, *pH 4.3*; 10 min
2. Rinse solution, pH 7.8; 1 min

3. Rinse solution, pH 7.8; 1 min
4. β-NADH solution, 45 min at 37°C
5. Distilled water, 30 s
6. ATPase incubation solution, pH 9.4; 30 min at 37°C
7. 1% $CaCl_2$, 30 s
8. 1% $CaCl_2$, 30 s
9. 1% $CaCl_2$, 30 s
10. 2% $CoCl_2$, 3 min
11. Distilled water, 30 s
12. Distilled water, 30 s
13. Distilled water, 30 s
14. Distilled water, 30 s
15. 2% ammonium sulfide, 3 min *(do this step under the hood)*
16. Running distilled water, 3 min
17. Hematoxylin, 5 min
18. Running distilled water, 3 min
19. 50% EtOH, 2 min
20. Drain and mount with glycerol gel

Results

β-Red fibers: Fibers that stain dark purple
α-Red fibers: Fibers that stain intermediate
α-White fibers: Fibers that stain light purple

Note

- For "cleaner" slides, filter the NADH solution before using it.
- If three fiber types are not identifiable, increase the pH of the acid pre-incubation solution until desired intensity is obtained.

Staining Sequence for Porcine

1. Acid pre-incubation solution, *pH 4.30*; 10 min
2. Rinse solution, pH 7.8; 1 min
3. Rinse solution, pH 7.8; 1 min
4. β-NADH solution, 45 min at 37°C
5. Distilled water, 30 s
6. ATPase incubation solution, pH 9.4; 30 min at 37°C
7. 1% $CaCl_2$, 30 s
8. 1% $CaCl_2$, 30 s
9. 1% $CaCl_2$, 30 s
10. 2% $CoCl_2$, 3 min
11. Distilled water, 30 s
12. Distilled water, 30 s
13. Distilled water, 30 s
14. Distilled water, 30 s

15. 2% ammonium sulfide, 3 min *(do this step under the hood)*
16. Running distilled water, 3 min
17. Hematoxylin, 5 min
18. Running distilled water, 3 min
19. 50% EtOH, 2 min
20. Drain and mount with glycerol gel

Results

β-Red fibers: Fibers that stain dark purple
α-Red fibers: Fibers that stain intermediate
α-White fibers: Fibers that stain light purple

Note

- For "cleaner" slides, filter the NADH solution before using it.
- If three fiber types are not identifiable, increase the pH of the acid pre-incubation solution until desired intensity is obtained.

Chemicals Needed for Staining Procedures

1. 2-amino-2-methyl-1-propanol
2. Acetic acid (glacial)
3. Aluminum ammonium sulfate
4. Ammonium sulfate
5. ATP
6. β-nicotinamide adenine dinucleotide
7. $CaCl_2$
8. $CoCl_2$
9. Ethyl alcohol
10. Glycerin (glycerol)
11. Fluoromount-G/glycerol gel(to mount slides)
12. HCl
13. Hematoxylin
14. KCl
15. Sodium Iodate
16. TNBT (tetranitro blue tetrazolium)
17. Tris base

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5 Converting Muscle to Meat: The Physiology of Rigor

Kirk W. Braden

Introduction

The quality of muscle foods relies heavily on the process of muscle to meat conversion. Antemortem and postmortem changes affect quality attributes such as tenderness, juiciness, and color. By ensuring that the process of muscle to meat conversion takes place under maximum positive conditions, the probability that the highest-quality product reaches the consumer can be increased.

When an animal is harvested, many changes occur at the cellular level of muscle. Upon exsanguination, approximately 50% of the animal's blood supply is removed. As normal life processes attempt to continue, the oxygen store present in the muscle is depleted. Depleted oxygen supply forces aerobic respiration to be superseded by the process of anaerobic glycolysis. Steady decrease in muscle pH from the normal homeostatic levels of 7.0–7.5 to a much lower 5.0–5.5 level occurs in response to the increased amount of lactic acid in muscle tissue as a result of anaerobic glycolysis.

Muscle function ultimately ceases as a result of the depletion of adenosine triphosphate (ATP) in muscle brought about by glycogen depletion as glycogen stores are converted to lactic acid by anaerobic glycolysis and falling pH renders glycolytic enzymes much less responsive. The ATP required to break the actomyosin bond is no longer available. Myosin heads therefore remain tightly bound to actin filaments creating stiffness in the muscle known as *rigor mortis*.

Onset of rigor will occur more rapidly if there are increased levels of lactic acid in the muscle directly antemortem, often resulting from increased stress. Inversely, rigor onset may be delayed if muscle glycogen stores are depleted antemortem, often resulting from increased physical activity for a prolonged period antemortem. Either situation will affect ultimate muscle pH and therefore rigor. Accompanying and contributing factors of rigor continue to effect tissue beyond rigor onset.

Muscle Metabolism upon Exsanguination

The living function of muscle can tell us much about the many changes that occur once an animal has been harvested and live muscle becomes consumable meat. In normal muscle contraction, a delicate balance of chemical matrices allows for muscle contraction and relaxation as normal muscle function is carried out. These homeostatic conditions are regulated by the endocrine and nervous systems. Muscle contraction and relaxation rely on energy provided by ATP. In living

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muscle, ATP is formed through a series of metabolic pathways. Glycogen stores are broken down to glucose molecules by the energy released in the dephosphorylation of two ATP molecules. These phosphorylated glucose molecules, along with glucose molecules available in the bloodstream, undergo glycolysis. Glycolysis generates two ATP of its own and passes two pyruvate molecules on to subsequent metabolic processes.

When an animal is harvested, chemical changes in normal muscle metabolism begin to occur at the point of immobilization. The alterations that now occur in normal metabolic pathways have the potential to change meat quality in the final consumed product. From the instant an animal is restrained for harvest, normal muscle function ceases to exist. It is important to minimize the time between restraint, immobilization, and exsanguination to normalize the transition between antemortem and postmortem metabolism.

When an animal is exanguinated, approximately 50% of their blood volume is lost as a result. Most of the remaining blood volume is removed with the viscera, and a negligible amount remains in the muscle tissue of the carcass. With the removal of blood, the continued oxygen source to muscle cells is also removed. The hemoglobin present in blood carries oxygen needed for normal metabolic processes to muscle cells. A certain amount of oxygen remains in muscle cells bound by myoglobin. Myoglobin has a greater affinity for oxygen than does hemoglobin and therefore retains oxygen in the muscle. This limited oxygen store allows glycolysis to continue followed by trichloro-acetic acid and oxidative phosphorylation through electron transport for the creation of ATP and the energy it provides for muscle relaxation. However, the oxygen store in the muscle is quickly exhausted and alternatives must be used to provide ATP. Some accessory ATP is created in a hydrolysis reaction metabolizing creatine phosphate (CP). CP is synthesized in the liver and is stored in the muscle in order to satisfy fluctuating energy demands. Nevertheless, these stores are also depleted as quickly as oxygen.

Once oxygen is fully depleted in the muscle, metabolism turns to anaerobic glycolysis, or glycolysis followed by fermentation. In anaerobic glycolysis, the pyruvate generated by glycolysis is broken down via fermentation to yield ATP, carbon dioxide, and lactic acid. Although there are ATPs generated by anaerobic glycolysis, it is significantly less efficient than aerobic metabolism yielding only 2 ATP compared to 36 in aerobic respiration. Therefore, the ATP generated by anaerobic means is quickly depleted by muscle relaxation mechanisms.

As oxygen supply is depleted by the loss of a functioning circulatory system, the ability of the muscle cells to eliminate waste also is lost. When anaerobic metabolism occurs in live muscle, such as in periods of stress or increased exercise or activity, the waste product of lactic acid is removed from the muscle tissue via the circulatory system and carried to the liver to be re-synthesized into glucose and glycogen, or to the heart to be metabolized to water and carbon dioxide (Aberle et al., 2001). With this waste removal mechanism gone, lactic acid continues to build up in the muscle until all glycogen stores have been exhausted.

As lactic acid accumulates in the muscle, muscle pH starts its gradual decline. Aerobic metabolism may be continuing in some cells while others in the same region have switched over to anaerobic means. The accumulation of lactic acid in the muscle tissue and associated drop in pH begins to render still functioning glycolytic enzymes nonresponsive and all manner of cellular metabolism and ATP production will eventually cease.

In the absence of ATP, cross-bridges formed by the binding of actin and myosin are unable to be resolved. Once calcium binds to troponin, troponin moves tropomyosin and myosin is able to bind with actin. The actomyosin bond creates a “cocking” action as neighboring muscle fibers slide into their contracted state. In the absence of ATP, energy generated as myosin splits ATP into ADP + P is missing and therefore unable to break the actomyosin bond. As a result, muscles remain in their

stiffened, contracted state. This prolonged period of inextensible muscle contraction is known as *rigor mortis* or the stiffness of death.

Rigor mortis proceeds in three stages. While some metabolic processes are still functioning in muscle and muscle extension can easily be obtained by applying force, an animal is in the delay phase of rigor mortis. As loss of ATP becomes limiting and muscle is becoming rigid, an animal is in the onset of rigor. Once muscles are completely inextensible, rigor has reached completion. Still not completely understood is the resolution of rigor. This is a period in which muscle flexibility begins to return, presumably due to damage to muscle proteins.

There are also other factors that can contribute to rigor and other postmortem muscle changes. The elimination of the circulatory system also means the loss of the regulatory mechanism for controlling muscle temperature. Deep muscle tissue cannot effectively dissipate heat in the absence of blood flow. Increased heat can speed metabolic reactions and thus shorten the time to achieve rigor. In addition, increased temperature coupled with rising lactic acid levels can attribute to unfavorable quality characteristics discussed later in this chapter.

Muscle fiber type also has an effect on postmortem muscle metabolism. Rates of metabolism differ between species and between muscle groups. Faster twitch white fibers generally metabolize faster and rely on glycolysis for energy production. Slower twitch red fibers metabolize slower and rely on oxidative metabolism. Accordingly, the faster twitch fibers will exhaust glycogen stores more readily and will proceed through the phases of rigor much faster than slower twitch fibers. Thus, muscle groups associated with locomotion enter rigor more quickly than muscle groups dedicated to support.

It is clear that the function of muscle metabolism plays a very important role in the conversion of muscle to meat. The processes and by-products thereof serve as effective means of monitoring the postmortem transition of animal to carcass. Additionally, the way in which the functional mechanisms of muscle cease after death and exsanguination play a significant role in the quality of the product yielded as an end result of harvest.

Development of Meat Quality

Several factors contribute to the eventual conversion of functioning live muscle tissue to meat and correspondingly have lasting impacts on the intrinsic quality properties of muscle-derived foods. Meat tenderness, color, texture, and moisture are drastically altered by aspects occurring during the development of early postmortem meat quality. Central to postmortem development of meat quality is temperature and pH effects that can be altered by many factors antemortem and postmortem. Quality attributes attained during the alteration process have lasting effects on fresh meat and meat utilized for further processed products. It is important to note that while control of factors affecting postmortem meat-quality attributes is of particular significance, manipulation of these factors can be difficult due to the extreme variation and quantity of these factors. Several of the factors implicated in the development of postmortem pH decline are discussed in this chapter.

The development of postmortem muscle pH in conjunction with temperature during and after rigor completion remains to be two of the leading factors in development of meat quality (Wulf and Page, 2000; Cavitt and Sams, 2003; Jensen et al., 2004; White et al., 2006). It is apparent from the previous discussion that a complex set of elements is set in progress upon exsanguination. In response to the loss of homeostatic control and the inability of muscle to receive oxygen, muscle glycolysis eventually becomes anaerobic, thus ultimately leading to the over-development of lactic acid. In response to loss of circulatory system function, the heat produced by the finite metabolism,

occurring immediately after exsanguination is unable to be dissipated via normal homeostatic regulation. Correspondingly, enzymatic reactions and glycolysis are hastened. The joint increase in temperature and decrease in pH, due to lactic acid build up trigger the beginning of the muscle to meat conversion.

Normal muscle pH regardless of species is approximated at 7.4 in living muscle (Aberle et al., 2001). With the upsurge of lactic acid due to postmortem anaerobic glycolysis, muscle pH begins to drop within 1 h postmortem. Increased temperature increases the rate of pH decline (White et al., 2004). Correspondingly, muscle pH values fall to levels of 5.4–5.7 after the resolution of rigor generally attained within 24 h postmortem. Normal meat pH after the resolution of rigor is generalized at 5.6–5.8. Perhaps more important is the rate of pH decline and thus the final pH achieved upon completion of rigor. A rapid pH decline immediately after exsanguination is linked to the development of a multitude of negative meat-quality attributes. Rapid pH decline generally leads to final pH levels much nearer the isoelectric point (5.1) of muscle. The isoelectric point can be generalized as the pH of net neutral charge or equal numbers of positive and negative charges. Muscle with a pH nearer to the isoelectric point, generally 5.2–5.4, suffers from watery, pale in color, and soft lean condition known as pale, soft, and exhudative (PSE). Inversely, a much slower rate of pH decline to levels, much higher than the isoelectric point, in the range of 6.6–6.9 leads to the converse condition in which meat is dry, dark in color, and firm. This condition is known as dark, firm, and dry (DFD). Either meat-quality condition is a departure from normal meat-quality characteristics and has lasting impacts on further development of meat-quality attributes, fresh meat appearance, and processed meat product values.

Beyond the development of visual meat-quality parameters, muscle pH and temperature significantly influence biochemical elements of muscle protein. Muscle proteins are increasingly denatured at higher temperature and pH levels. Protein denaturation and correspondingly protein proteolysis are key factors in the development of the typical meat-quality conditions. As proteins are subjected to elevated denaturation, a loss of protein functionality is seen. Similar only in general appearance to PSE and DFD, denatured proteins begin to lack the ability to hold water. The lack of water-binding capacity is accompanied by the loss of protein solubility and binding. The concerted effect of reduced water and protein binding pose significant barriers in processed meat production and these elements will be further discussed in the following section of this chapter as well as in Chapter 7 (Water-Holding Capacity of Meat). Additionally, protein denaturation lends to functionally and visually significant changes in muscle pigments. Muscle pigment and equally meat color is of considerable importance in fresh meat production and sales, as well as, in processed meat production.

Water-Holding Capacity

The moisture content of muscle and consequently meat plays a vital role in muscle food palatability, functionality, and shelf-life as muscle is composed of approximately 75% water. Upon exsanguination, several homeostatic processes are altered leading ultimately to the rise in lactic acid. As discussed previously, the decline in muscle pH drastically alters the water-holding capacity (WHC) of meat. As pH departs from the isoelectric point (above or below 5.1), the amount of charge (positive or negative) increases and correspondingly the available charges for WHC increases. Thus, WHC increases as muscle pH deviates from the isoelectric point. It is however quite uncommon for muscle pH to fall below the isoelectric point and thus as muscle pH nears the isoelectric point from 7.4, WHC is decreased as in the case of the classical condition PSE. Inversely, WHC is quite

markedly increased in DFD meat due to an over-abundance of available charge at high meat pH levels of 6.6–6.9.

Water in muscle can be divided into three general types: bound, immobilized, and free. Bound water is held tightly via myofibrillar protein charges. Immobilized water is found within the muscle ultrastructure, but is not bound to myofibrillar proteins as in the case of bound water. Immobilized water accounts for the largest portion of muscle bound water. Rather immobilized water is sequestered within muscle cell membranes and attracted to bound water. Free water is held within muscle by weak capillary force. As muscle pH nears the isoelectric point, the ability for water, a dipolar molecule, to be held within the charged myofibrillar protein structure is diminished. Additionally, with the onset of rigor muscle continues to contract without relaxation ultimately due to the loss of CP and the corresponding lack of re-phosphorylation of ADP to ATP. This contraction without relaxation that occurs during the development of rigor reduces the amount of cellular space for water. This rigid contracted structure leads to diminished WHC. Diminished space conjoined with gradual destruction of cellular membranes by pH, temperature, and protein denaturation leads to release of immobilized water. Immobilized water is then diverted into the sarcoplasm (Jensen et al., 2004). Accumulated water in the sarcoplasm is markedly less restricted and is retained in the cell structure primarily via cellular membranes and capillary forces.

Several antemortem and postmortem factors can contribute ultimately to the WHC of meat. Factors such as genetics, antemortem production practices, carcass chilling, and electrical stimulation (ES) play key roles in the conversion of muscle tissue to meat and more specifically WHC. Genetic predispositions such as the halothane (*HAL*) gene or the association with porcine stress syndrome (PSS) and the Rendement Napole (RN) gene are commonly linked to the development of negative meat-quality attributes and reduced WHC. Antemortem factors such as feed withdraw, stress, and nutritional plane also play significant roles in WHC of meat. The integral role the WHC of meat plays in meat quality and muscle foods is discussed in depth in Chapter 7—Water-Holding Capacity of Meat.

Meat Color

Color is perhaps the most critical component of fresh meat appearance, and more importantly a consumer's perception of meat quality is strongly influenced by product appearance. Meat color and eventual discoloration of meat is a combined function of (a) muscle pH; (b) antioxidant status; (c) oxidation of muscle pigments; and (d) oxidation of lipids. Primary fresh meat pigments consist of deoxymyoglobin, oxymyoglobin, and metmyoglobin, all of which are a variant of oxygen state of the nonprotein heme ring of myoglobin. Muscle pH decline and ultimate muscle pH have considerable effects on muscle-color attributes. As muscle pH declines from homeostatic levels postexsanguination, muscle membranes and myofibrillar proteins are increasingly denatured. Increased protein denaturation leads to a loss of color intensity. Enzymatic reactions are accelerated by way of increased muscle temperature postexsanguination, and the remaining available oxygen is consumed. Accordingly, temperature reduction after harvest can slow enzymatic reaction and anaerobic glycolysis ultimately reserving the remaining available oxygen. Conversely, the abnormal condition DFD results in a high relative muscle pH in which oxygen consuming enzyme activity is increased and diminishes the amount of available oxygen to convert deoxymyoglobin to its red oxygenated (oxymyoglobin) state. Additionally, muscle pH and ultimately the development of lactic acid/lactate play a central role in metmyoglobin reduction via lactate dehydrogenase (Mancini and

Ramanathan, 2007). Lactic acid accumulation in muscle may also interact with myoglobin and increase stability by myoglobin redox (Mancini and Ramanathan, 2007).

Normal color development in meat occurs by way of several elements of which not all are solely related to muscle pigment state. Normal color in meat is characterized by a postmortem muscle pH of 5.6–5.8, a species that is relatively bright red in color, firm, and has a non-exudative lean surface. Muscle pH, the role pH plays in water binding, and the degree of water binding are central factors in the development of meat color. The importance of postmortem muscle pH cannot be overstated as abnormal meat quality/color conditions such as PSE and DFD occur mainly due to atypical postmortem pH upon completion of rigor mortis. The pale color of PSE meat is characterized by a greater than normal proportion of free water. The proportion of free water not contained within muscle cells is referred to as extracellular water (Aberle et al., 2001). Increased amounts of free water located between muscle cells and on the surface of cut muscle acts as a multitude of reflective surfaces thus the lean surface appears light in color. Conversely, the dark lean color associated with DFD lean is contributed to increased amounts of bound water as greater amount of water is held within cellular space. High amounts of intracellular water reduces the amount of reflective surfaces and light is absorbed into the lean cut surface leading to a markedly darker lean color in comparison with normal meat color.

The development of meat color can be affected by several ante- and postmortem factors. Animal diet, genetics, and postmortem temperature control have lasting impacts on meat color. For instance, diet composition and withdrawal of feedstuffs prior to harvest affect glycogen storage and thus postmortem muscle pH. Diets high in energy commonly increase subcutaneous fat cover or back fat, and hence chilling rate can be decreased. The effects of pH and temperature have been discussed previously. Additionally, diets high in supplemental or naturally occurring antioxidants serve to decrease muscle pigment oxidation. Meat color development and factors effecting meat color are further discussed in the Chapter 9—Meat Color.

Aging and Tenderness

The process of rigor development has lasting impacts on the development or lack of meat tenderness. Subsequently, the aging process, defined as extended time of storage beyond the resolution of rigor, plays a vital role in the emergence of meat palatability and quality parameters. Specifically, meat tenderness is drastically altered from the point of exsanguination to aging.

Perhaps most importantly, the primary changes in meat tenderness occur in response to Z-disk disruption. Due to muscle super-contracture without relaxation, as found in high-voltage, electrically stimulated carcasses, sarcomere length is drastically reduced by the irreversible formation of the actomyosin complex. The shortening of the sarcomere has profound effect on meat tenderness due to the increase in bulk density of muscle fibers. However, during rigor resolution, sarcomere length is again altered via degradation of the Z-disk. As discussed in Chapter 2, the Z-disk is responsible for maintaining several portions of the sarcomere, the least of which is providing a myofibrillar protein scaffold and registry. Degradation of the Z-disks begins prior to any other myofibrillar protein proteolysis. Continual degradation of the Z-disk gives rise to increased fracture of the muscle filament registry. Correspondingly, the tension developed during rigor and the super-contracture formation of the actomyson complex propagates the continual rupture of the myofibril from the Z-disk. The process of myofibrillar rupture from the Z-disk creates myofibrillar fragments, thus reducing bulk density across the sarcomere and thus the muscle. This reduction consequently increases meat tenderness.

Several postmortem cooperative elements affect tenderness development in meat of which postmortem anaerobic glycolysis, subsequent pH decline, muscle fiber type, and myofibrillar protein proteolysis are of particular importance. The rate of postmortem glycolysis and thus pH decline can affect changes in meat tenderness (O'Halloran et al., 1997; White et al., 2004) and can be influenced by the level and rate of cooling during the onset of rigor (Locker and Hagyard, 1963). It has been found that an integral relationship between pH/temperature/time and their subsequent effects on postmortem muscle glycolysis give rise to the dynamic nature of tenderness development (White et al., 2004). Postmortem pH decline within the initial stages of rigor development plays a key role in tenderization of muscle due to the activity of certain pH-dependent proteases, and inconsistency in pH postmortem gives rise to inconsistency in tenderness (O'Halloran et al., 1997). Skeletal muscle proteases are integrally linked to the development of postmortem muscle tenderness. Several muscle proteases have been implicated in postmortem muscle proteolysis including the calcium-dependent proteases (CDP) or calpains, cathepsins, and serine-dependent proteases (SDP). The three classifications of muscle proteases should be further evaluated based upon the ability to elicit effects on the elements of the sarcomere most commonly associated with the development of tenderness. The SDP and cathepsins lack the ability to degrade elements and are either considered crucial to muscle tenderness, as in the case of SDP, or are not sequestered within cell organelles and cannot be utilized, as in the case of cathepsins. SDP are not highly active in myofibrillar proteins and are not highly associated with myofibrillar proteins implicated in tenderness. The lysosomal constriction of cathepsins does not facilitate their release and thus contact with myofibrillar proteins is limited. However, the CDP are responsible for a majority of the postmortem protein proteolysis and degradation accrued during the aging process. The CDP is composed of two main enzymes: m-calpain, requiring molar amounts of calcium to be activated, and μ-calpain, requiring micromolar amounts of calcium for activation. Protein proteolysis and loss of muscle ultrastructure during the development of rigor will be further discussed in the "Postmortem Factors" section of this chapter. The development of protein and connective tissue tenderness is additionally addressed in Chapter 4—Tenderness.

Antemortem Factors

To further facilitate the understanding of the conversion of muscle to meat and the subsequent physiology of rigor, the abundance of associated antemortem factors must be explored. The production of meat animals is a complex process of which elements across a continuum from farm to fork are involved. Among the antemortem items implicated in the development of meat quality after exsanguination are genetics of the meat animal, diet consumed during finishing, environmental interaction, and occurrence of physiological stress. Each of the aforementioned items can have lasting influences on the development of meat quality and consequently on the fresh meat or meat utilized for further processed food/meat production.

Genetics

Meat-quality traits across a range of quality attributes are moderately heritable. It is not uncommon for selection of important meat quantity and composition attributes to negatively affect pertinent meat-quality and palatability attributes. For instance, the genetic selections for increased lean meat yield as in the case of callipye or double-muscled conditions. However, these selection criteria

commonly come with negative associations to meat quality, as meat from animals expressing the callipyge phenotype lacks consistency in tenderness. Some negative artifacts can be seen in the genetic conditions of HAL and RN carriers.

Partially in response to halothane anesthesia, the Halothane condition has been classified. Animals, more specifically pigs, carrying the homozygous recessive condition for the *HAL* gene are stress susceptible and are predisposed to PSS. The Halothane condition maps to chromosome 6 (Rehfeldt et al., 2004; te Pas et al., 2004). Additionally, a mutation in the porcine ryanodine receptor (RyR) has been found to be responsible for the Halothane condition (Fugii et al., 1991). The mutated receptor encodes for an altered calcium channel that resides in the sarcoplasmic reticulum. The altered calcium channel protein lacks complete functionality, thus a higher proportion of sarcoplasmic reticulum calcium is released into the adjacent sarcomere. As greater amounts of calcium are released into the muscle, the speed of rigor development and formation of the actomyosin complex is increased. The development of rigor occurs much earlier in the postmortem period than normally apparent. Correspondingly, postmortem metabolism is increased to restore the relaxed state of muscle and subsequently greater proportions of lactic acid are formed during early stages of the onset of rigor. Jointly, as pH declines, early postmortem temperature dissipation is at a minimum due to loss of blood supply and lack of chilling. The addition of relatively low pH and increased temperature leads to increased levels of postmortem protein denaturation as previously discussed and the abnormal meat-quality condition PSE commonly results.

The occurrence of another genetic mutation in porcine is commonly referred to as Rendement Napole or the RN condition. The RN condition was identified in porcine (Milan et al., 2000) and is found almost exclusively in the Hampshire breed. Consequently, the RN condition was first termed the "Hampshire Effect" as it was first noticed in France that in meat from Hampshire pigs generally had a markedly lower pH when compared to other contemporaries (te Pas et al., 2004).

The RN condition is known to be a single dominant gene mutation (Navieu, 1986) in which RN-positive pigs produce approximately 70% greater glycogen in large white glycolytic fibers when compared to non-RN pigs. The RN condition has been mapped to chromosome 15 in pigs (Mariani et al., 1996; Milan et al., 1996). Yet another affect of the *RN* gene is carcass lean characteristics, as the RN allele has been associated with leaner carcasses.

The RN condition results in the development of an altered adenosine monophosphate (AMP) kinase cellular protein. AMP kinase is a cellular protein responsible for regulation of glycogen synthesis. The altered nature of AMP kinase ineffectively functions as a feedback mechanism for glycogen production and correspondingly greater amounts of glycogen are synthesized. Subsequently, following a normal pH decline via production of lactic acid, the heightened levels of glycogen are converted to excess lactic acid in anaerobic glycolysis after exsanguination. Thus, the meat from RN pigs is characterized by a much lower pH postmortem and the ensuing quality attributes are applied.

The relationship between muscle fiber type composition and eventual meat quality cannot be overlooked. Muscle fiber type and the respective characteristics, more specifically the biochemical elements of metabolism, associated with specific fiber types are linked with several elements of the conversion of muscle to meat and the accompanying development of rigor (Maltin et al., 1997; Klont et al., 1998; Karlsson et al., 1999). A majority of the meat-quality attributes linked to effects of muscle fiber type center around oxidative versus glycolytic predominant metabolism type and the subsequent pH decline in muscle. It becomes relevant to evaluate muscle fiber type impacts on meat quality as myofibrillar composition is of moderate-to-high heritability (Dietl et al., 1993). Through intense genetic selection, it is possible to effect changes on meat quality via muscle fiber types (Larzul et al., 1997). It is common in porcine production that lean content and percentage of

glycolytic fibers are positively correlated (Ashmore, 1974; Klosowska et al., 1985). Additionally, a link between sensorial qualities of meat and fiber type (Crouse et al., 1991) is evident with large fast glycolytic fibers negatively correlated to tenderness and slow oxidative fibers positively correlated.

Muscle fiber types differ in metabolic, biochemical, and biophysical characteristics such as glycolytic and oxidative metabolism capacities, shear fiber diameter, fiber color, and content of substrates, such as glycogen and lipids (Schiaffino and Reggiani, 1996; Klont et al., 1998; Karlsson et al., 1999). Three primary fiber types emerge based upon the isolation technique of myosin ATPase staining. These fiber types consist of (1) Type I fibers that are red in appearance, slow twitch in contractile action, function with a mostly oxidative metabolism, and are highly fatigue-resistant; (2) Type IIB fibers that are lighter to white in appearance, fast twitch in contractile action, function with a primarily glycolytic metabolism, and are easily fatigued; and (3) Type IIA fibers that are red in appearance and intermediate to Type I and IIB fibers with an oxidative or glycolytic metabolism. Type IIB fibers are fast twitch in contractile state and moderately fatigue-resistant. A fourth subpopulation of Type IIB fibers has been classified as Type IIX with similar biochemical and biophysical properties. Muscle fiber type and their corresponding properties are additionally discussed with greater emphasis in Chapter 3—Muscle Structure and Myofibrillar Proteins.

The possibilities of muscle fiber type genetic selection for modulation of meat-quality attributes are apparent. The pork industry, through intense selection for lean muscled market animals, has steadily selected for an increase in the proportion of large white fast fibers which are more glycolytic and thus demand more oxygen. Type IIB fibers consequentially succumb to anaerobic glycolysis at a much higher rate, thus producing excess lactic acid as opposed to all other fiber types (Crouse et al., 1991; Karlsson et al., 1999). Conversely, Type I and IIB fibers are found to be more desirable. Oxidative fibers impart a higher ultimate pH, more intense darker color, increased WHC, and thus increased tenderness (Chang et al., 2003). It is evident that fiber type composition is an important indirect factor in ultimate meat quality and the most favorable fiber types expressing a predominantly oxidative metabolism (Chang et al., 2003).

In respect to meat animal stress events, glycolytic fibers are substantially more susceptible. Glycolytic fibers require a greater overall volume of oxygen in relation to oxidative fibers, thus in episode of heightened stress, oxygen capacity is diminished. In response to preharvest stress, the diminished oxidative capacity impairs the glycogen reserves and glycolysis thus producing greater amounts of lactic acid. The effects of lactic acid increase and pH decline have been well discussed in the “Development of Meat Quality” section of this chapter.

Stress and Diet

The maintenance of homeostasis is of paramount importance in living organisms and more specifically animals. Several body processes are superseded to maintain homeostatic condition in regard to stimuli. Numerous environmental factors can act as external stimuli and thus encourage animal excitability or more succinctly termed animal stress. It is evident that several environmental factors, that food animals are exposed to, exist and that these factors have lasting impacts on meat quality. Environmental factors can include confinement conditions, ambient temperature, relative humidity, sound, and length of day or light exposure. Nonetheless, environmental factors are not solely responsible for an animal’s responses to external stimuli rather the animal, specific factors are also partially accountable. Animal factors that can contribute to the stress response to external environmental stimuli are species, age, sex, stress resistance, and genetics. Perhaps one of the most important elements of an animal’s response to external stimuli is the respective animals’ stress

susceptibility. It has been previously stated, as the case of PSS, that certain genetic conditions are predisposed to elevated or heightened stress states thus producing the corresponding meat-quality issues.

When an animal perceives an environmental stimulus to be antagonistic to the maintenance of homeostasis, several biochemical elements are altered. Mainly, metabolism is drastically altered in response to stress, via a complex arrangement of hormonal regulation. Upon excitation, the animal's hypothalamic–pituitary–adrenal (HPA) axis (Stephens, 1980; Grandin, 1995; Schaefer et al., 2001) is activated. Activation of the HPA axis has significant indirect eventual effects on meat quality (Schaefer et al., 2001). Upon activation of the HPA axis, the hypothalamus is stimulated to release corticotrophin releasing hormone (CRH) which triggers the release of adrenal corticotrophin hormone (ACTH). ACTH in turn elicits effect on the adrenal cortex and adrenal medulla to release glucocorticoids and catecholamines. Of greater importance is the glucocorticoid cortisol and the catecholamine epinephrine/norepinephrine. In respect to conversion of muscle to meat and the effects of stress, cortisol and epinephrine are key regulating factors. Cortisol is partially responsible for upregulation in gluconeogenesis, proteolysis of muscle tissue, and subsequent lipolysis of adipose tissue. The increased need for metabolic substrates and the corresponding action of cortisol is evident. Additionally, epinephrine and norepinephrine stimulate several biological processes. Epinephrine effectively increases heart rate, blood pressure, and free fatty acid levels. Moreover, glucogenolysis/glycolysis, lipolysis, and metabolic rate are hastened via epinephrine and norepinephrine. Epinephrine, by way of effect on the aforementioned biological processes, is able to rapidly increase the mobilization of glucose in response to external stress stimuli and a perceived need to maintain homeostasis. As energy mobilization is increased, epinephrine and norepinephrine act synergistically in the body by increasing blood flow to transport products for metabolism and waste products of metabolic processes.

The stress response of animals and the corresponding upregulation in metabolic pathways apply several elements of change on meat quality. Increased muscle metabolism due to stress increases heat output and thus increases oxygen consumption by further increasing enzymatic reactions. Stress and the corresponding release of the associated hormones emphasize the anaerobic pathway of glycolysis as oxygen demands for aerobic glycolysis are not met due to diminished oxygen capacity. As anaerobic metabolism predominates, the corresponding products are synthesized. Thus, through increased anaerobic metabolism, a greater proportion of lactic acid is generated. This is especially evident in large white Type IIB muscle fibers. However, it is possible to abate lactic acid build-up via the blood stream by transport to the liver. This clearing process, however useful, takes time; thus, in the case of preharvest stress, lactic acid abatement is minimal. Excess lactic acid accumulation in muscle and thus meat had been discussed thoroughly in the "Development of Meat Quality" section of this chapter.

Many elements of meat quality such as color, WHC, and texture are affected by preharvest stress. In terms of meat quality, the major metabolic changes, as influenced by preharvest stress, are depletion of glycogen and consequent inability of muscle to develop adequate acidity levels postmortem (Gregory and Grandin, 1998). Inadequate acidity levels postmortem prevent muscle from reaching normal muscle pH (5.6–5.8) upon resolution of rigor and the DFD condition is commonly seen. Several antemortem stressors have been related to meat-quality defects PSE (Rosenvald and Andersen, 2003) and DFD (Apple et al., 1995). Perhaps more specifically affected are live/carcass weights, quality grades, and rate of downgrades due to DFD or PSE occurrence (Schaefer et al., 2001).

The effect of nutritional plane on animal reaction mechanisms when stress is encountered and the resultant meat quality is significant. Diet and recuperation time can effectively replenish glycogen stores poststress event. Muscle glycogen levels increase with elevated metabolizable energy intake

(Pethick et al., 2000) and increased glycogen stores can act as a reserve, ultimately functioning as a buffer in the preharvest period. Two major factors contributing to suboptimal glycogen levels at harvest are excessive glycogen depletion from stress during transport and lairage (Jones and Tong, 1989) and poor nutritional plane on the farm (Pethick and Rowe, 1996). The feeding of an adequate nutritional plane in food animal production to increase glycogen buffer, along with carefully managed transport and lairage, should assist in the production of a more consistent high-quality product (Lowe et al., 2002).

Postmortem Factors

Developments of meat quality occurring throughout the postmortem period, beginning with exsanguination, are of extreme importance when evaluating all possible elements affecting the conversion of muscle to meat. Primary postmortem factors contributing to meat quality consist of protein proteolysis, temperature/chilling, and ES. Each factor is involved with the biochemical nature of muscle as a live tissue and the succeeding transition process. A substantial amount of time and research has been employed by industry and scientific communities to ascertain the exact roles of each of the aforementioned factors. However, the complete nature of their interaction and the role each plays in the development of meat-quality attributes is still not clear. Nevertheless, it is evident that each factor acts as a noteworthy factor in postmortem meat-quality emergence with special attention to postmortem tenderization of meat.

Protein Proteolysis

Proteolysis occurring after exsanguination is vital to the improvement of meat tenderness after rigor. As discussed, in relation to rigor development, the process of rigor resolution is partially elicited by the proteolysis of proteins associated with the Z-disks of the sarcomere. This proteolysis lends to the fracture of myofibrils and thus myofibers. There is increasing evidence that selected myofibrillar proteins are denatured in the early postmortem period and proteolysis ultimately leads to a reduction in muscle tension (toughness) (Davey and Gilbert, 1966; Bandman and Zdanis, 1988; Hopkins and Taylor, 2004). The degradation of certain myofibrillar proteins is partially attributed to a set of proteolytic enzymes that will be further discussed shortly. When evaluating the tenderization of meat, it is important to remember that a majority of the changes in tenderness occur due to myofibrillar protein degradation and not integral changes in muscle connective tissue. Quite inversely, muscle connective tissue is relatively stable in the postmortem period (McCormick, 1994; Hopkins and Taylor, 2004).

Several elements occurring in the postmortem period contribute specifically to protein proteolysis. Among the factors contributing to postmortem proteolysis, temperature, pH, and the extent of contracture during rigor are significantly important. However, the role of postmortem tenderization of meat is also dependent upon species (Dransfield et al., 1981; Etherington et al., 1987). Approximately 80% of the postmortem tenderization of pork is complete within the first 5 days postmortem, while similar levels of tenderization in beef take a minimum of 14 days (Koohmaraie et al., 1991).

Temperature during postmortem storage has the ability to drastically alter the extent of tenderization by affecting the rate of enzymatic reactions occurring in the muscle. Higher temperatures generally increase enzymatic reaction speed and lower temperatures do the inverse. Conversely, temperature is not readily controlled across the entire carcass as deep muscles, closer to the center of the

carcass, will have much slower heat dissipation when chilled. Additionally, the extent of contracture during rigor development undoubtedly affects the ultimate tenderization of meat. Muscles with a greater tension, developed during rigor, are quite likely to be less tender in comparison to those experiencing less tension development. This effect is apparent even after postmortem proteolysis. Furthermore, pH levels have been shown to affect protein denaturation postmortem as a fast rate of pH decline is linked to the development of more tender meat (Claeys et al., 2001; Rees et al., 2003).

Several proteolytic skeletal muscle enzyme groups are associated with postmortem proteolysis. These enzyme families consist of the CDP (calpains), cathepsins, and SDP. When evaluating the effectiveness of these enzyme families, in terms of meat tenderization, the calpain family is predominantly responsible for a majority of the changes in meat tenderness.

Calpains

Calpain activity in meat was first discovered by Olson et al. (1977). Calpains have been classified throughout the recent past beginning with their discovery in 1977. Thus, the current classification of calpains is segmented into two primary types: μ-calpain and m-calpain. Several types of calpains, such as p94 (Hopkins and Taylor, 2004), have been identified; however, in terms of proteolysis and meat tenderization, the two main types of calpains are the aforesaid. Classification of calpains is chiefly based upon the activation level of Ca^{2+} ions needed for functionality. μ-Calpain requires micro-molar levels of Ca^{2+} ions for activation, whereas m-calpain requires much greater (molar) amounts for activation. Due to the necessity of Ca^{2+} ions for activation, the rate of postmortem proteolysis by calpains is quite variable. Perhaps even more critical is the presence of the endogenous inhibitor of calpains known as calpastatin (Murachi et al., 1981). Calpastatin levels are quite variable across several animal-related elements; thus as calpastatin levels increase, the postmortem tenderization of meat due to calpain proteolysis activity decreases. Even so, calpains function is maximized *in vitro* at pH 7.5 and at temperature 25°C (Hopkins and Taylor, 2004). Clearly, the development of rigor, subsequent pH decline, and general postmortem meat storage practices (mainly temperature of storage) do not lend to maximum calpain activity. However, calpain function postmortem is vital to the postmortem meat tenderization. The elements of calpain function and tenderness are further discussed in Chapter 6—Tenderness.

Cathepsins

The function cathepsins perform in postmortem tenderization of meat is not well known. Due to the location of cathepsins in the lysosomes (Goll et al., 1983) they must be released to affect myofibrillar degradation. Subsequently, the release of cathepsins is impeded by exsanguination and the loss of cellular function. Nonetheless, the failure of lysosomal membranes, partially due to falling pH postmortem, allows minimal cathepsins release (Pommier et al., 1987). Consequently, cathepsins play a minimal part in postmortem proteolysis.

Muscle Ultrastructure

A complex system of biochemical processes occur during postmortem storage leading to myofibrillar fragmentation and proteolytic activity that has been demonstrated as a key component. Proteolytic activity is centered around the degradation of the structural protein elements associated with the Z-disks registry. Several studies indicate that a majority of the protein degradation postmortem is targeted at titin, nebulin, troponin-t, desmin, filamin, and vinculin in terms of tenderness development (Taylor et al., 1995). Specific protein degradation, protein tenderness, and connective tissue tenderness are discussed with greater emphasis in Chapter 6—Tenderness.

Temperature and Chilling

The temperature at which muscle undergoes the transition through rigor plays an integral role in the quality exhibited by the resulting meat. Production and dissipation of heat must be balanced at a favorable level to ensure that postmortem muscle metabolism will not lead to unfavorable conditions within the muscle. The muscle's premier heat dissipation system is lost when the animal is bled and blood no longer circulates to promote temperature control. Elevated muscle heat coupled with falling pH due to lactic acid production can lead to conditions such as PSE. Elevated heat equals faster metabolism which results in depleted oxygen that leads to anaerobic glycolysis and lactic acid build up. Lactic acid build up leads to falling pH which can lead to increased free water when pH falls below the isoelectric point. Greater amounts of free water and free calcium resulting from ceased muscle contraction leads to a higher degree of protein degradation (Lawrie and Ledward, 2006).

It is favorable to reduce the temperature of the carcass as quickly as possible to reduce the occurrence of microbial growth. However, it is possible that chilling muscle too quickly may result in unfavorable conditions such as cold shortening and thaw rigor. These conditions result when muscle tissue is chilled below 15–16°C or frozen before the completion of rigor (Aberle et al., 1994). When temperature comes above 15–16°C, contraction results as Ca^{2+} released into the sarcoplasm and remaining ATP resulting from incomplete rigor allow severe cross-bridges to form. Muscle can be affected by a shortening of fibers by 60–80% (Aberle et al., 1994). The shortening results in water loss and severe toughening.

Maintaining muscle at favorable temperatures during the process of rigor is of utmost importance. Cooling carcasses too slowly or too quickly can result in damage to the quality of meat. Consequently, special care must be taken to ensure the cooling process to be favorable for all aspects of meat production for quality and safety.

Electrical Stimulation

Of the many postmortem factors affecting meat quality, ES may be the factor which is easiest to manipulate and control. First discovered in 1749 when Benjamin Franklin noticed that ES of turkeys immediately postmortem had tenderizing effects on their flesh (Lawrie and Ledward, 2006), ES has now become commonplace in the American abattoir.

ES is utilized as a measure by which rigor onset and completion is expedited. In postmortem muscle, as discussed earlier in this chapter, metabolism continues until a point where muscle oxygen and ATP stores are fully depleted. Normally, this process would take a number of hours. ES allows continued impulses to be sent to muscle, postmortem, for muscle contraction. Voltages ranging from 30 to 3600 are commonly used for ES (Aberle et al., 1994). Higher voltages promote stimulus of the muscle directly, whereas lower voltages rely on message sent by the nervous system to continue stimulated muscle contraction. This prolonged muscle activity more rapidly depletes energy stores and hastens the progression to anaerobic glycolysis. Consequentially, expedited anaerobic glycolysis more rapidly induces lactic acid build up and pH decline, thus shortening the time required to reach the completion of rigor mortis.

Favorable effects of ES include prevention of cold shortening, improved muscle color and quality grade, increased muscle firmness, and increased meat tenderness. ES speeds the process of rigor development and allows carcasses to be rapidly chilled to a point which is favorable for microbiological protection without exceeding time and temperature constraints that can lead to cold shortening.

Cold shortening occurs when muscle reaches a temperature below 10°C with a pH above 6 while the muscle still contains ATP stores. When this occurs, serious tenderness problems can result. Accelerated glycolysis brought about by ES depletes any ATP stores and resulting lactic acid build up drops muscle pH below specified levels, therefore negating the phenomenon of cold shortening and its effects on muscle tenderness. Cold shortening is not considered to be as detrimental in pork as it is in beef and lamb. Pork muscle, on average, contains a larger amount of white, fast-twitch fibers which tend to carry out glycolysis normally at a much faster rate than the predominant red, slow-twitch fibers found in beef and lamb muscle. Thus, the pork industry has much less need for ES.

Improved lean color can be seen in carcasses that have undergone ES. This is presumably due to an increased amount of extracellular water induced by expedited pH decline. This increase is only enough to enhance reflectivity and therefore color and does not exceed the point where the cut surface would become exhudative (Lawrie and Ledward, 2006). Brighter red lean color may lead to the increased definition of appearance of intramuscular fat. It is because of this visual attribute that beef plants employing ES often see in increase in the number of cattle grading USDA Choice or an increase of approximately 5% (Romans et al., 1994). It was this favorable marketing tool that sparked widespread commercial use of ES.

The debate over the effects of ES on meat tenderness continues. While some believe it to be simply an artifact of the assured prevention of cold shortening, still others believe that there are lasting effects on meat tenderness resulting from increased proteolytic activity resulting from high levels of Ca^{2+} released in repeated severe muscle contractions induced by ES (Aberle et al., 1994). Still another theory stems from the physical disruption of muscle fibers that may, indeed, be caused by violent muscle contraction during ES. In either event, improvements in meat tenderness rely heavily on the inherent tenderness of the individual animal and/or muscle group depending on deviating intrinsic factors discussed in the "Antemortem Factors" section in this chapter.

Although there are several positive outcomes of ES, over-stimulation can result in detrimental effects in the realm of meat quality. Geesink et al. (2001) found that mild stimulation of beef carcasses produced favorable quality and tenderness attributes. However, they also found that additional or intense stimulation caused decreased tenderness and quality attributes in comparison with control groups. For this reason, ES and its employment in the commercial abattoir may need to be closely monitored to ensure maximum positive outcome.

Analysis of Muscle pH

Given the close relationship of lactic acid build up and pH decline to rigor progression, intramuscular pH measurement is perhaps the best indicator of muscle to meat conversion. However, due to the tedious and time-consuming nature of obtaining the reading, commercial monitoring of intramuscular pH does not occur until long after carcasses have reached the chill cooler and often at periods nearly 24 h postmortem. The findings of Stahl et al. (2006) suggest a measurement of intramuscular pH taken within 1 h postmortem.

Taking the Measurement

1. Make a deep puncture incision, 5–6 cm deep from the exterior surface of the carcass with a clean, sharp knife at the area of the 12th and 13th ribs for beef and the 10th and 11th ribs for pork and lamb. This incision may be used for repeated readings throughout the duration of the study.

2. Insert pH instrument probe and allow for the reading to become stable in accordance with the manufacturer's instructions. Record the reading.

It is favorable to use a glass-tipped KCl pH probe for the most precise measurements. The probe should be calibrated before pH readings are taken and again between equal numbers of animals within the sample group. Calibration buffers, equilibrated to the temperature of the sample, should be used that closely reflect the expected readings to be encountered (pH 4 and 7) in order to obtain accurate readings.

Analysis of Sarcomere Length

Sarcomere length determination is an excellent indicator of muscle tenderness and rigor development. With the use of procedures outlined in Cross et al. (1981) and Koolmees et al. (1986), sarcomere measurements can be used to evaluate the progress of rigor and detect any unfavorable outcomes such as cold shortening or thaw rigor.

Solutions

(Koolmees et al., 1986)

0.1 M $NaHPO_4$ Buffer at pH 7.2

	1 L	2 L
Na_2HPO_4 (MW = 141.96)	10.18 g	20.36 g
NaH_2PO_4 (MW = 137.99)	3.91 g	7.82 g

Dissolve in distilled water, pH and bring up to volume.
Store at 4°C.

5% Glutaraldehyde in 0.1 M $NaHPO_4$ buffer at pH 7.2

Glutaraldehyde (25%) 200 mL. Bring up to 1 L with 0.1 M $NaHPO_4$ buffer. Store at 4°C.

0.2 M Sucrose in 0.1 M $NaHPO_4$ Buffer at pH 7.2

	1 L	2 L
Sucrose	68.46 g	136.92 g

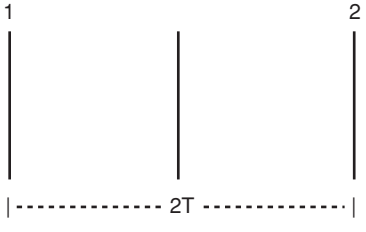
Dissolve and bring up to volume with 0.1 M $NaHPO_4$ buffer.
Store at 4°C.

Preparation of Muscle for Laser Diffraction

1. Excise, in triplicate, small pieces (3.0 × 3.0 × 2.0 cm) of muscle with the fibers running longitudinally. Place the cores in scintillation vials. Add 5% glutaraldehyde solution and fix for 4 h at 4°C.
2. Pour off the glutaraldehyde solution and replace with the 0.2 M sucrose solution. Fix overnight at 4°C. Cores can be stored at 4°C for up to 7 days in this solution.
3. Individual fibers are tweezed from samples and places between a clean microscope slide and a cover glass with a drop of sucrose solution to prevent drying.

Sarcomere Length Determination

1. The fiber on the slide should be placed directly in the beam of a 2.0 mW monochromatic beam of light generated by a helium neon gas laser tube (Cross et al., 1981).
2. A diffraction pattern will be projected on the screen as follows:



The distance between bands 1 and 2 is equal to 2T in the following formula.
$T = 1/2$ the distance from 1 to 2.
Measure from 1 to 2 in millimeters and then divide by 2.

3. Sarcomere length is determined by the following equation (Cross et al., 1981)

$$\mu = \frac{0.6328 \times D \times \sqrt{\frac{T^2}{D} + 1}}{T} \tag{5.1}$$

where D, distance from specimen to diffraction pattern screen in millimeters (preferably 100 mm); T, spacing between diffraction bands in millimters and $0.6328 = 632.8$ (the wavelength of the laser) $\times\ 10^{-3}$.

It is important that an adequate number of muscle excisions are taken from each animal ($\geq$6) and that a significant number of fibers are tested from each excision sample ($\geq$2) to ensure that enough sarcomeres are measured from each animal ($\geq$12) to provide results that are statistically sound.

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6 Meat Tenderness

Chris R. Kerth

Introduction

Of all the quality parameters that we find in meat, tenderness is perhaps the most complex. For something that at first seems like a simple "tough" or "tender", we soon discover that accurately describing tenderness is actually quite difficult. Descriptors like chewy, hard, tough, stringy, soft, or mushy all describe the presence or lack of tenderness. We will also find that objectively measuring tenderness is very difficult because a machine cannot take into consideration all of these different textural characteristics. While certain parameters of tenderness may be measured accurately, other textural traits may not be reflected. As a matter of fact, the human machine is usually the best comprehensive method for determining tenderness.

What is Tenderness?

The most common way to express tenderness is resistance to tooth pressure, or in other words, how much force is required to bite through a piece of meat. While this is accurate, it only describes one part of tenderness. It can also be described by hardness, firmness, ease of fragmentation, and numerous textural definitions such as springy, mushy, mealy, or crumbly. In freshly cooked meat, the two most common measurements are resistance to tooth pressure, and ease of fragmentation. Often, these are referred to as initial and sustained tenderness, as measured by the initial bite (force to bite through) and how long it takes to fragment the piece (chewiness). As we discuss the complexity of tenderness, we can generally divide tenderness into three categories: protein tenderness, connective tissue amount, and background effects. Protein tenderness is usually determined by two factors in the muscle: sarcomere contractile state and amount of myofibrillar protein degradation. The amount of connective tissue is self-explanatory and background effects are those that indirectly influence tenderness, such as juiciness and flavor, which can be determined by atmosphere, cooking method, intramuscular fat, and degree of doneness (Whipple et al., 1990a).

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Factors that Affect Tenderness

Sarcomere Contractile State

As described in Chapter 5, the process of rigor results in permanent cross-bridges being formed between myosin and actin filaments in the sarcomere, which causes the muscle to become stiff. The sarcomere length can vary from about 1.5 microns, which is the width of the thick filament with the Z disks touching either end of the myosin, to about 2.7 microns, which is a fully extended sarcomere with little overlap of the thick and thin filaments. The state of contraction of the sarcomere is primarily determined by the physical restraint or stretching applied to each specific muscle as the carcass is hung during harvest and the muscles go through rigor. Some muscles, like the semitendinosus, are stretched as the hind leg is pulled out behind the pelvis when it is hung by the Achilles tendon. The femur acts like a lever, with the pelvic ball socket serving as the fulcrum to stretch that muscle. At the same time, the longissimus muscle is on the opposite side of the lever (femur) and becomes compressed without any mechanical restraint applied to it during rigor. The result is that the longissimus muscle has relatively short sarcomeres by comparison.

Additionally, the biochemical environment inside the muscle fiber during the process of rigor can dramatically affect the final state of sarcomere contraction. All muscles go through a process called rigor shortening (Fig. 6.1). As discussed in Chapter 5 regarding the process of rigor, the last step in the contraction of muscle is for the myosin head to attach to the thin filament and bend, which slides the thin filament, shortening the sarcomere, then release from the thin filament with the binding of an ATP. If no ATP is present (as in the case of rigor completion), then the myosin head stays

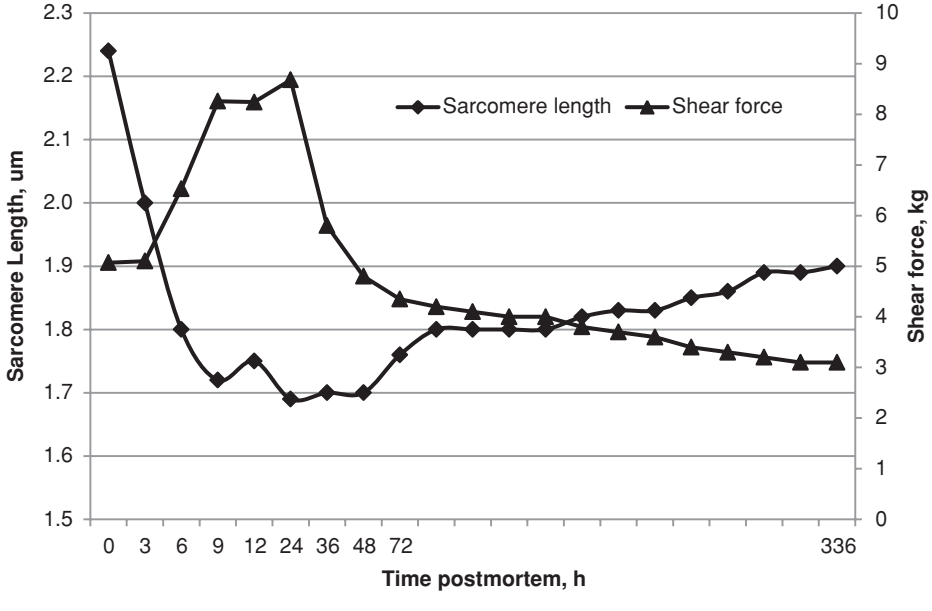

Figure 6.1 As the sarcomere shortens early postmortem because of rigor shortening, shear force increases indicating a direct relationship between the sarcomere length and tenderness. This can be overcome after a short period of time by the proteolytic action of endogenous enzymes like the calpains. In the absence of protein degradation postmortem, meat tenderness is largely influenced by the sarcomere length. (Adapted from Koohmaraie et al., 1996).

attached to the thin filament and the last step becomes "bending" resulting in a slight shortening of the sarcomere before the completion of rigor. As long as ATP is present, the sarcomere can shorten as much as physical forces applied against it allow. If conditions exist where excess cellular calcium is present when ATP is also present, excess sarcomere shortening results; this is because the excess calcium triggers the mechanism that uncovers the myosin-binding sites. This can happen when a muscle is chilled rapidly and the sarcoplasmic reticulum is unable to retain the stored calcium. The result is a condition called cold shortening and results in muscle that has shorter-than-normal sarcomeres.

Research has shown that with all else being equal, the shorter the sarcomere, the more force required to shear through the muscle (Weaver et al., 2008). The reason is that shorter sarcomeres have much more overlap of thick and thin filaments, with many more actomyosin crossbridges being formed, and a sarcomere that is much denser compared to an extended sarcomere. Therefore, any measure that can be taken prior to the completion of rigor that results in longer sarcomeres will help to produce meat that is more tender (Weaver et al., 2009).

Electrical stimulation applied to the carcass after exsanguinations but prior to the carcass entering the cooler helps to prevent cold shortening. The electricity produces muscle contraction, which uses ATP. The goal then is to use up as much of the ATP as possible before the meat enters the cooler, since the cold temperatures result in calcium being released from the sarcoplasmic reticulum. Naturally, this calcium will be released regardless of other events, but the idea is to not have both ATP and calcium in the muscle at the same time. Electrical stimulation "burns" ATP prior to the calcium being released and effectively reduces the chance of cold shortening of sarcomeres.

Another method termed the Texas A&M Tenderstretch, is useful in changing sarcomere length. After the carcass is eviscerated and split, the carcass side is hung from the rail by the pelvis at the aitchbone instead of the Achilles tendon as is traditionally done. This allows the weight of the round to pull the hind leg down, and with the pelvis serving as the fulcrum, stretches the longissimus muscle, lengthening the sarcomeres. Others have shown that by cutting through the thoracic vertebrae between the 12^{th} and 13^{th} ribs, but leaving the longissimus muscle intact after the carcass is split and washed in the abattoir, allows the weight of the forequarter to pull on the longissimus muscle, lengthening the sarcomeres.

Myofibrillar Protein Degradation

Perhaps the most widely studied trait related to tenderness is the degradation of myofibrillar proteins during postmortem aging of meat (Etherington, 1984; Koohmaraie et al., 1991). As discussed earlier, the myofibrillar proteins are the structural proteins in the muscle that are responsible for contraction. These proteins are arranged in an extremely complex and highly organized manner that allows them to carry out these functions. For instance, how the thin filaments are organized—six thin filaments surround each thick filament in a parallel orientation, and then these thin filaments are attached to the Z disk, which allow the pulling forces in one sarcomere to be coupled with the pulling forces in the adjoining sarcomere—demonstrates the complex arrangement of these structural proteins. Because of the mechanical function of these proteins necessary to carry out muscle contraction and the accompanying tension placed on muscle, ligaments, tendons, and bones, it is very important that the physical structure of the muscle be very strong. Unfortunately, the strength of this structure in the muscle works against us if we are trying to create a meat product that is tender. To have a tender piece of meat, we need a muscle with a very weak structure that allows the meat to break apart easily when we eat it. To weaken the protein structure postmortem, we need to have enzymes

that are specific to proteins and break down these proteins. Because they work to degrade protein, these enzymes are called proteolytic enzymes, and there are numerous types of these enzymes that occur naturally within the muscle.

When myofibrils are viewed under magnification at various times postmortem, one of the first things that becomes apparent is that the Z disks seem to gradually fade from 0 to 21 days postmortem (Taylor et al., 1995a). It was first hypothesized that α-actinin, the primary protein of the Z disk, was degraded since this prominent structure of the sarcomere clearly disappeared. It was also hypothesized that, because of the importance of the thick and thin filaments along with the permanent actomyosin crossbridges that form at rigor, actin and myosin may also be degraded by these proteolytic enzymes. Research has since shown that none of these proteins undergo significant degradation postmortem. We now know that the proteins that are most susceptible to postmortem degradation are titin and nebulin (especially where they join the thick and thin filaments to the Z disk), as well as several of the intermediate and costameric proteins like desmin, vinculin and dystrophin, and troponin T. While most of these proteins are structural in nature, troponin T is a regulatory protein found on the thin filament that is responsible for moving tropomyosin from covering the myosin-binding sites. So, troponin T actually has little or no direct effect on the structural stability of the sarcomere, but it is a very good indicator of the total amount of proteolysis in the muscle. In fact, the most common measure of specific protein degradation using SDS-PAGE and western blotting is the appearance of a 30 kDa protein, a product of troponin T degradation.

Most of these proteins that are known to break down in the presence of these endogenous enzymes are, in fact, located at or near the Z disk of the sarcomere. The two gap proteins titin and nebulin have been shown to anchor myosin and actin filaments, respectively, to the Z disk. Additionally, intermediate and costameric filaments lay perpendicular to the length of the myofibril and serve to anchor one myofibril to another or to the sarcoplasm. Consequently, the breakdown of these filaments would certainly have a detrimental impact on the structural integrity of the myofibril, resulting in less force required to break the muscle apart, making the meat more tender.

The endogenous enzymes that are known to work on myofibrillar proteins fall into two basic types: the cathepsins and the calpains (Whipple and Koohmaraie, 1991). The cathepsins are found in the lysosomes, which are organelles, with the interior of the lysosome having a very acidic environment (Koohmaraie and Kretchmar, 1990). The cathepsins, including cathepsin B, D, H, and L, were some the first enzymes thought to be responsible for breaking down proteins during postmortem aging of meat since they tend to be active in acidic environments and the pH of meat declines to about 5.5 after rigor, thereby creating a suitable environment for these enzymes to hydrolyze proteins (Whipple et al., 1990a, 1990b; Sentandreu et al., 2002). It was later discovered that it is unlikely that these enzymes are actually released from the lysosomes. Additionally, cysteine proteinase (of which cathepsins are also classified) inhibitors called cystatins are found in various cellular compartments with an apparent function to inhibit proteolysis by these enzymes in the cytosol or extracellular space. This would likely at least partly inhibit their function if they were released from the lysosome. Furthermore, when myofibrils are incubated with purified cathepsin enzymes, myosin and actin tend to be degraded fairly rapidly, again indicating that they are most likely not responsible for the tenderizing effect of aging, as these proteins are not degraded *in vivo*.

In the late 1980s, a new classification of enzymes called the "calcium-dependent factor" or "calcium-dependent proteases" were discovered (Koohmaraie, 1988) and later termed "calpains" with two basic types—μ-calpain and m-calpain (for review, see Goll et al., 1992, 2003; Koohmaraie and Geesink, 2006). These two types are classified on the concentration of calcium required to activate them, with μ- and m-calpain being activated by low and high levels of calcium, respectively.

Because calcium is no longer able to be sequestered in the sarcoplasmic reticulum postmortem, the intracellular concentration of calcium rises and activates the calpains. It is widely thought that the level of calcium in postmortem muscle is not high enough to activate m-calpain, so μ-calpain is the primary enzyme responsible for protein degradation postmortem (Geesink et al., 2006). The calpains also have a specific inhibitor, calpastatin. Calpastatin is also found in the sarcoplasm and has been found to be a key component in myofibrillar protein turnover and meat tenderness. Numerous antemortem factors can elevate the activity of calpastatin. Growth implants, oral growth promotants, and genetics such as *Bos indicus* in beef and callipyge in sheep may cause calpastatin activity to increase. This results in a decrease in protein degradation, and usually an increase in muscle growth in the live animal, but less tender meat postmortem. Many of these factors can be managed to get the benefits in growth from the live animal without impacting tenderness caused by protein degradation.

While other proteases can be found in muscle tissue, their effects on myofibrillar protein degradation have been shown to be mixed at best. This includes the proteosomes (Koohmaraie, 1992; Taylor et al., 1995b; Robert et al., 1999; Goll et al., 2008), and the caspases (Sentandreu et al., 2002; Ouali et al., 2006). Protein degradation is perhaps the factor that is most carefully managed in trying to produce meat that is tender. Usually 7 to 14 days of postmortem aging, with as much as 35 days, is used to give the enzymes time to break down the myofibrillar proteins. After all, at the refrigerated temperatures in which meat is stored, enzyme activity is slowed significantly. Today, we have EPDs for genetic selection and even genetic markers to test for tenderness which is directly related to the level of enzyme activity and protein degradation.

Connective Tissue

As we discussed in Chapter 3, collagen, by its very biochemical nature, is an extremely strong tissue. With three basic layers of collagen around the muscle (epimysium), muscle bundles (perimysium), and muscle fibers (endomysium), it is distributed throughout the muscle tissue and gives the muscle its strong but elastic structure. When considering collagen or connective tissue as it relates to tenderness, there are two basic properties that we must consider: type and amount. The type of connective tissue is related to its chemical makeup or structure and therefore is an indicator of its strength and solubility. The amount of connective tissue refers only to the quantity of connective tissue found in a particular muscle.

The type, or structure, of the connective tissue found in meat is important as it determines the basic solubility traits of the tissue. For example, elastin is well known to be extremely tough, insoluble with heat, and is generally found in tissues that need extreme strength. It has also been isolated in perimysial and endomysial tissues in meat, although in much lower quantities than in blood vessels, for example. More important is the structure and related solubility of collagen. Unmodified collagen fibers by themselves tend to be fairly soluble upon heating. Where their strength and insolubility comes from is in posttranslational modification of the collagen fibers. The most common of these is in either intramolecular (between α chains within the same molecule) or intermolecular cross-linking. The three types of cross-linking found in muscle collagen are all covalent in nature: disulfide bonds formed between cysteine residues in type III and IV collagen, divalent bonds formed between residues of lysine or hydroxylysine aldehydes, and complex bonds between two or more collagen chains arising during the aging of collagen. Cross-links that are intramolecular in nature have little effect on the solubility, and therefore on tenderness, as they are not involved in increasing the stability of the collagen fiber, only the tropocollagen molecule. The cross-links that are of most

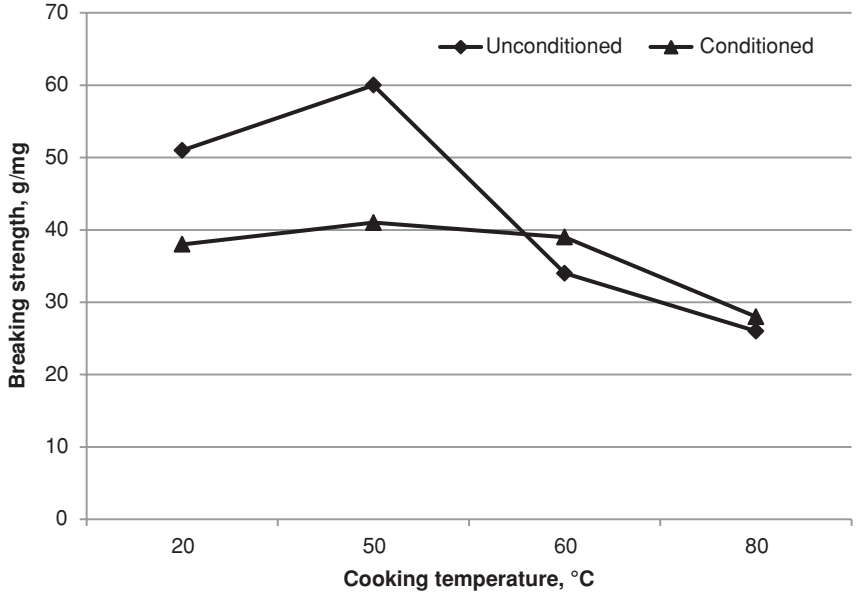

Figure 6.2 Tensile breaking strength (g/mg) of isolated perimysia taken 24 h postmortem from the semitendinosus muscle of 18-month-old heifers that had been either frozen at −20°C immediately (unconditioned), or stored for 14 days at 1°C, then frozen (conditioned). Samples were thawed, and cooked to the indicated temperature to illustrate the effect of conditioning and thermal processing on perimysium (collagen) strength. (Re-drawn from Lewis et al., 1991)

concern are those complex intermolecular bonds that arise from age. While these bonds are not well understood, glycosylation of the lysine residues and an increase in collagen fiber diameter are thought to be contributors to the decrease in collagen solubility as the animal ages. See Lepetit, 2008 and Purslow, 2005, for review of collagen structure and its role in meat quality.

As collagen is heated, as is done during the cooking process, it shrinks and turns from a solid to a liquid gelatin. The amount of complex cross-links among collagen fibers determines the ability of the collagen to solubilize or "melt" (Fig. 6.2). As the animal ages and more of these cross-links form, the collagen shrinks, but is unable to change from a solid state, causing the meat to be tough (Figs. 6.3 and 6.4). Additionally, the type and amount of all of the cross-links determine the degree of shrinking and the temperature at which the collagen solubilizes.

The total amount of collagen found in the muscle tissue also plays a large role in the tenderness of meat. Naturally, the amount of epimysial tissue is not critical as this outer layer can be trimmed from the muscle and not consumed. The amount of perimysial and endomysial tissue is important, as these are found within the muscle and cannot be removed. The biggest difference that we find in the amount of collagen is between muscles within the animal. As a general rule, muscles that are used for locomotion tend to have higher quantities of collagen, while muscles used for support or for very finely adjusted movements (as for the eye) tend to be very low in collagen content. Support muscles that we commonly think of include the psoas major and longissimus muscles. These muscles are very low in collagen and therefore the tenderness of these muscles is determined mostly by the degree of protein tenderness.

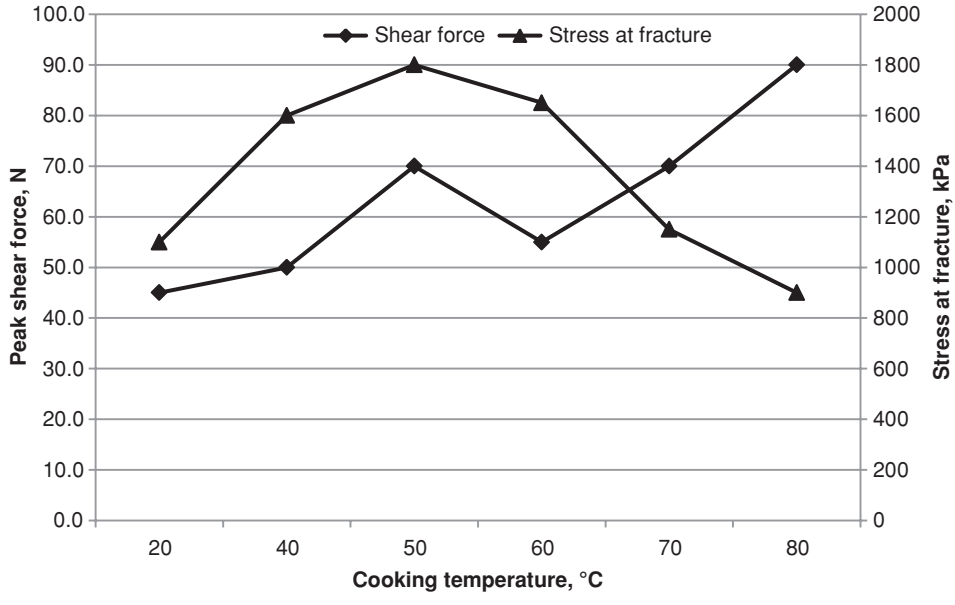

Figure 6.3 The effect of cooking temperature end point on the Warner–Bratzler shear peak force of whole beef semitendinosus and the tensile breaking strength of perimysial connective tissue of beef semitendinosus cooked to different temperatures. (Re-drawn from figures in Christensen et al., 2000)

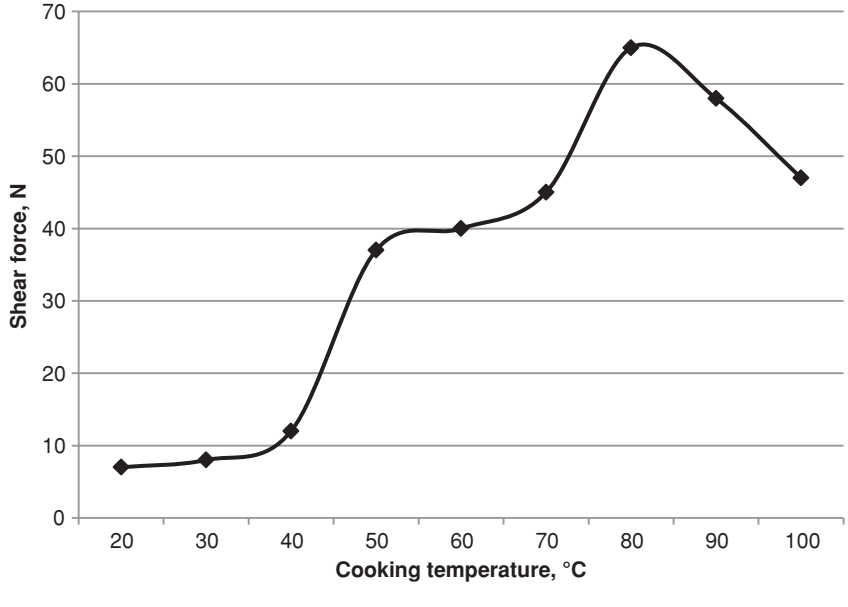

Figure 6.4 The two-phase effect of cooking temperature on shear-force values. Two distinct toughening phases are shown, one between 40 and 50°C, the other between 70 and 80°C. They are clearly separated and are a reflection of distinctly different events occurring within the tissue. An increase in tenderness (reduction in shear force) from 80 to 100°C reflects another cooking event from the dissolution of connective tissue. (Re-drawn from Davey and Gilbert, 1974)

Background Effect

In discussing factors that affect tenderness thus far, we have been able to objectively describe the factors that influence the tenderness of meat. We must also be aware that factors that influence tenderness and which are not as directly involved exist. We lump these into a category called "background effects". These are factors that indirectly influence a person's ability to judge tenderness, and therefore should be discussed and accounted for.

The greatest of these factors is marbling or intramuscular fat. Much debate has centered around the influence of marbling on tenderness. For the most part, we can say that marbling has little direct influence on tenderness (Wheeler et al., 1994). Nevertheless, a number of theories exist suggesting that marbling may directly impact tenderness. The "bulk density" theory states that since fat tissue is less dense than lean tissue (about 0.9 g/cm^3 vs. 1.1 g/cm^3), if there is more intramuscular fat, the meat will be less dense and easier to bite through. Another is "lubrication," which theorizes that the intramuscular fat serves as a lubricant that makes it easier for a person's teeth to bite through a piece of meat. The most probable is the "background effect" that suggests that because marbling increases juiciness and flavor traits of meat, marbling indirectly improves tenderness. In other words, a piece of meat with more marbling will have more juice and better flavor compared to meat with little marbling, and the improvement of these other palatability traits improves the *perceived* tenderness of the meat.

Research has shown that marbling has little direct effect on objective measurements of tenderness like shear force. In fact, it only accounts for about 10% of the total variation in tenderness. There is still a general trend for shear force measurements to decrease (increased tenderness) with increasing amounts of marbling. More than anything, marbling should be considered an insurance policy, and that as the amount of marbling increases, juiciness, and flavor increase and the variation in tenderness tends to decrease. In other words, it is possible to have a very tender piece of meat with little marbling, but the chance of meat being tender improves as marbling improves. Finally, marbling helps to insure eating quality regardless of cooking. As the degree of doneness increases to medium well (~80°C) or well done (~90°C), the meat will tend to dry out from a loss of water. Marbling helps to insure that juiciness and flavor remain even at these higher cooking temperatures. Savell and Cross (1988) reported a "Window of Acceptability" for marbling with at least "Slight" marbling required for minimum acceptability and no appreciable increase in palatability above "Modest" marbling.

Measuring Tenderness

Because of its overwhelming importance in determining the consumer acceptance of meat products, reliable and repeatable measures of tenderness and the factors that are associated with tenderness are needed. We will focus on instrumental measures of tenderness and in later chapters will discuss using humans to measure this complex sensory trait.

Shear Force

The most common and widely accepted objective measure of tenderness is shear force. As the name implies, it is a measure of the amount of force required to cut through, or shear, a piece of cooked meat. To insure uniformity, all samples are cut into pieces of uniform dimension. Steaks or chops are cut 2.5 cm (1 in) thick and cooked using a dry-heat cookery method (see Chapter 10—Cookery).

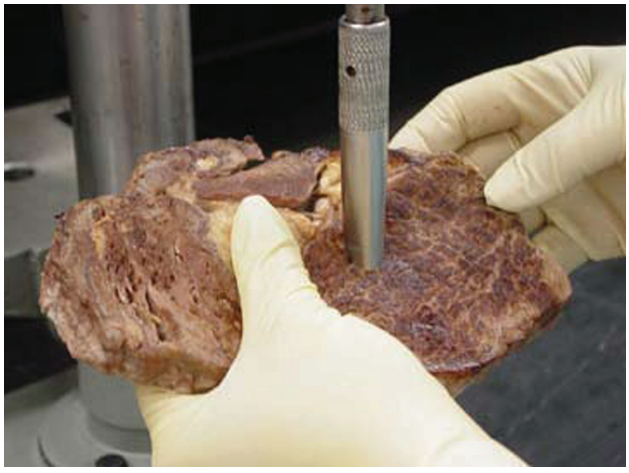

Figure 6.5 After determining the direction of the muscle fibers in the cooked and cooled steak, a 1.27-cm-diameter core is placed parallel to the muscle fibers and slowly turned while applying pressure to remove the core for the Warner–Bratzler shear force.

The cooked meat is then cooled to allow accurate sizing of the samples to be used for shearing. Research has shown that the shear force of cooked versus cooked-then-cooled meat is not different. The commonly used method, Warner–Bratzler shear force, requires the removal of round cores, usually 1.27 cm (0.5 in) in diameter. A total of six cores must be used to accurately characterize the tenderness of a muscle. If the chop is small, several chops must be used to obtain the six cores. The cores should be removed parallel to the muscle fiber orientation so that when the core is sheared in half, the blade shears directly perpendicular to the muscle fibers (Fig. 6.5). The cores are then placed in either a Warner–Bratzler shear machine or a computerized device with a load cell. A V-shaped blade of specified thickness with a specifically rounded blade is attached to a scale or load cell and mechanically shears through the meat core (Fig. 6.6). A measurement of the peak force required to shear completely through the core is read from the machine and recorded. An average of the peak shear force for all six cores is used to characterize the tenderness of the muscle. The computerized version of the shear machine is also able to calculate the total area under the curve of shear force in addition to the peak force. This further characterizes the tenderness of the meat.

More recently, a method has been developed in which a 1-inch-thick slice of meat is removed from the cooked steak or chop parallel to the muscle fiber orientation (Fig. 6.7). This single slice of meat is then sheared in the same manner as for the cores, except that the blade used for slice shear force is straight instead of V-shaped (Fig. 6.8). A single slice shear measurement is required and has been shown to be at least as, or more repeatable than the Warner–Bratzler method.

In general, shear force measurements are a good measure of protein tenderness. In other words, they do not do a good job describing the differences in textural properties when connective tissue amount differs. Therefore, shear force is mostly used in cuts low in connective tissue, especially using the longissimus muscle.

Sarcomere Length

Certainly, the length from Z disk to Z disk of sarcomeres in muscle is critical to understand differences in tenderness of meat. The difficulty is that the typical sarcomere ranges in length from

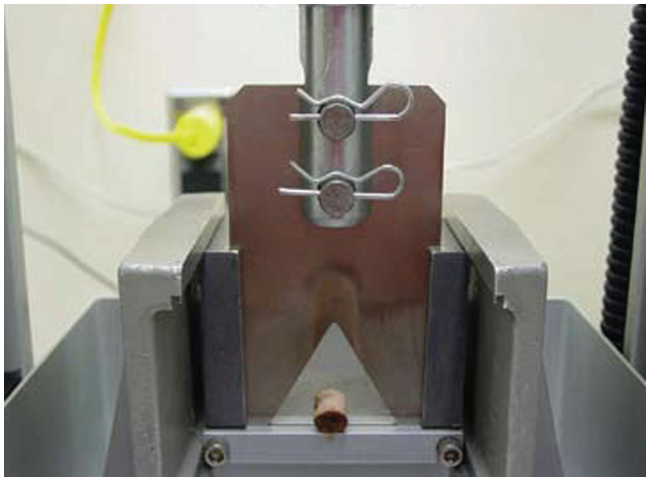

Figure 6.6 The Warner–Bratzler shear machine uses a V-shaped blade with a specific angle and edge. Force is applied downward on the blade which is attached to an electronic load cell which will measure the force (expressed in kg or N) required to shear through a 1.27-cm-thick round core one time and perpendicular to the length of the muscle fibers.

about 1.5 μm for a fully compressed sarcomere with thick filaments touching the Z disks, to about 2.7 μm for a fully extended sarcomere. The extremely small distances make microscopic methods of measuring sarcomere length very difficult and would introduce a large amount of experimental error in the measurements. A much better method for measuring sarcomeres utilizes a laser beam. A single muscle fiber is removed from a muscle sample that has been fixed in a solution of glutaraldehyde, and then placed on a slide in the path of the laser beam. When the laser hits the Z disk of the

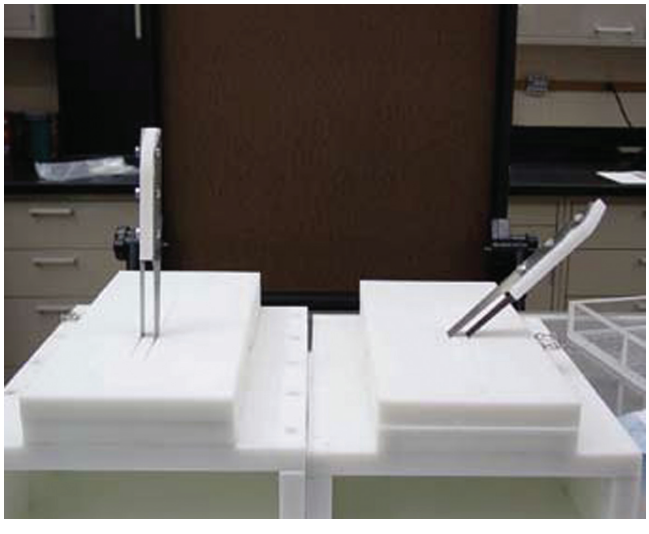

Figure 6.7 Slice shear is a rapid method of removing a sample from a cooked steak for shear testing. Specialized double-bladed knives are constructed and used in a jig that places the steak at the correct angle for removing the sample parallel to the length of the muscle fiber. The jig on the right is for the *Longissimus muscle* that has been removed from the 12^{th} to the 13^{th} rib. At this place on the carcass the muscle fibers run at a 45^{o} angle to the flat surface of the steak.

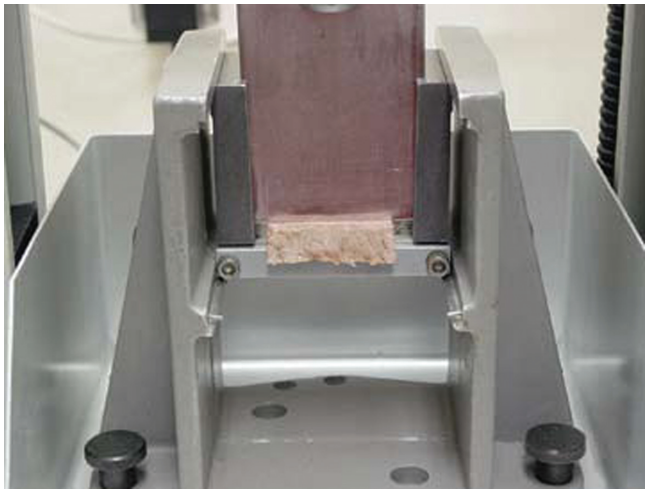

Figure 6.8 For slice shear force, a flat blade is attached to the load cell on the shear machine to measure the force (expressed in kg or N) required to shear through the slice of meat one time and parallel to the length of the muscle fibers.

sarcomere, the Z disk refracts the laser and creates laser lines. The distance between the laser lines measured a fixed distance from the muscle fiber can be measured in millimeters and put into an equation that gives sarcomere length. The procedure is easy and very repeatable.

Protein Degradation

Just as with measuring sarcomere length, quantifying protein degradation can help to describe differences that exist in tenderness. This can be especially important because animals are usually harvested at very similar ages, so connective tissue is not different and at harvest, the carcasses are all hung the same, so sarcomere length may not differ significantly. Therefore, protein degradation may be one of the most important factors that we measure related to meat tenderness.

Many times, a crude measure of total myofibrillar protein degradation is all that is needed to describe differences in tenderness. For that, myofibrillar fragmentation index (MFI) is a good measure of total protein degradation. Muscle samples are homogenized in a standard salt solution (SSS) then centrifuged seven times to get a somewhat crude purification of the myofibrillar proteins. Once they are purified and suspended in solution, a protein concentration is determined using a colorimetric determination, then a solution of 0.5 mg/mL of the sample protein is prepared and read in a spectrophotometer at 540 nm. The absorbance is multiplied by 200 to obtain an MFI reading. The higher the MFI value, the more degraded the protein. The basic theory of this test is that if the total protein concentration suspended in solution is constant, then differences in absorbance are caused by differences in the length of the proteins and protein fragments. The more fragmented the proteins, the greater the amount of light absorbed.

A much more specific measure of protein degradation is the use of SDS-PAGE and western blotting. Gel electrophoresis separates each of the individual myofibrillar proteins along with their degradation products. If a known concentration of protein is loaded onto the gel, then each of the bands on the gel can be quantified based on their relative intensity. This can be done visually, or with computer imaging software that converts the bands to pixels, when scanned into a computer and can then analyze the pixels to quantify the density of each band. Therefore, as bands get less

intense, it is a sign that protein is being degraded. Additionally, if bands become more intense, those bands may be indicative of degradation products of whole proteins. A good example of this is the analysis of troponin T and the 30 kDa protein. Through western blotting, antibodies against troponin T protein were used to stain the troponin T protein. When all of the proteins of a gel are transferred to blotting paper and stained with the anti-troponin T antibodies, both the troponin T band as well as the 30 kDa protein band stained, indicating that the 30 kDa protein was in fact a fragment of the original troponin T protein.

Connective Tissue

Just as in protein tenderness, a general measure of the amount and type of connective tissue in a muscle is critical to understanding any differences in tenderness caused by connective tissue. The most basic measure is that of total collagen as well as heat-soluble collagen. These two measures give general quantification to the two most important factors in connective tissue that we have described—type and amount.

A more sensitive and descriptive method for determining the total amount of connective tissue in the muscle is to actually measure the thickness of the perimysium and/or endomysium. By staining 20 μm thick slices of muscle tissue and then staining them with picric acid to stain the collagen red, the tissue can be viewed on a microscope. The thickness of the perimysium at 40 × or 100 × power is sufficient to accurately measure the thickness of this connective tissue layer in the muscle. This test may be more sensitive than measuring the total amount of collagen and better describes differences that exist within the muscle.

MFI Procedure (Myofibrillar Fragmentation Index)

1. From each of three locations across the muscle sample, scissor mince ~1/3 of 4 g. The 4 g sample should be free of visible connective tissue and fat. Run samples in duplicate.
2. Put sample in an Eberbach blender and add 40 mL of 2°C MFI buffer. Homogenize on high for 30 sec.
3. Pour into 50 mL centrifuge tubes with lids.
   Centrifuge at 1000 × g for 15 min at 2°C. Discard the supernatant.
4. Re-suspend the pellet in 40 mL of MFI buffer using a stir rod.
   Centrifuge at 1000 × g for 15 min at 2°C. Discard the supernatant.
5. Add 10 mL of MFI buffer to the pellet and vortex until well mixed.
6. Pour through a polyethylene strainer to remove the connective tissue. Rinse through the strainer with an additional 10 mL of MFI buffer.
7. Pipette 0.25 mL of suspension into 13 × 100 glass tubes.
8. Add 0.75 mL of MFI buffer.
9. Add 4 mL of biuret reagent and vortex.
10. Put in the dark at room temperature for 30 min. (BSA standards should be run at the same time).
11. Read the absorbance at 540 nm.
12. Calculate mg/mL of protein in the suspension.
13. In a 13 × 100 glass tube, make 8 mL of a 0.5 mg/mL dilution of each sample.
14. Vortex and shake well immediately before reading the absorbance at 540 nm. A B&L Spec 20 with a large slit width should be used.
15. Multiply absorbance reading by 200.

MFI buffer (100 nm KCl, 20 mM K-phosphate (pH 7), 1 mM EGTA, 1 mM $MgCl_2$, and 1 mM NaN_3)

	2 L	4 L	6 L	8 L	10 L
KCl	14.91 g	29.82 g	44.73 g	59.64 g	74.6 g
KH_2PO_4	2.72 g	5.44 g	8.16 g	10.88 g	13.6 g
K_2HPO_4	3.5 g	7.0 g	10.5 g	14.0 g	17.5 g
EGTA (MW 380.4)	0.76 g	1.52 g	2.28 g	3.04 g	3.8 g
$MgCl_2$	0.41 g	0.82 g	1.23 g	1.64 g	2.1 g
NaN_3	0.13 g	0.26 g	0.39 g	0.52 g	0.65 g

Dissolve in deionized distilled water (pH 7.0) and bring to volume.

Biuret reagent (Check in liquid chemical cabinet for pre-made reagent first!)

Dissolve 1.5 g cupric sulfate ($CuSO_4 \cdot 5H_2O$) and 6 g sodium potassium tartrate (Rochelle salt, $NaKC_4H_4O_6 \cdot 4H_2O$) in about 500 mL double distilled water in a 1000 mL flask. To this, add (with constant stirring) 300 mL of freshly prepared, carbonate free 10% NaOH. Make up to 1 L with double distilled water and store in a brown polyethylene bottle. Discard if a black or red precipitate appears.

Purified myofibrils Preparation of purified myofibrils using differential centrifugation (Source: Goll et al., 1974)

Procedure:

1. Ground or minced meat: (If you use less than 4 g of sample, use 50 mL conicals, otherwise use 50 mL centrifuge tubes).
   a. Suspend in 10 volumes (v/w) standard salt solution by homogenizing for 10 s. Depending on volume, use polytron or blender.
   b. Centrifuge at 1000 × g for 10 min.
2. Discard supernatant (contains CAF & other sarcoplasmic proteins), but be careful not to discard the "fat cap".
   a. Re-suspend pellet in 6 volumes (v/w) of standard salt solution by homogenizing for 10 s.
   b. Centrifuge at 1000 × g for 10 min.
3. Discard supernatant, but be careful not to discard the "fat cap".
   a. Re-suspend pellet in 8 volumes (v/w) of standard salt solution by homogenizing for 10 s.
   b. Pass suspension through nylon net strainer. (Tupperware)
   c. Centrifuge at 1000 × g for 10 min.
4. Discard supernatant, but be careful not to discard the "fat cap".
   a. Re-suspend pellet in 8 volumes (v/w) of standard salt solution by homogenizing for 10 sec.
   b. Pass suspension through nylon net strainer.
   c. Centrifuge at 1000 × g for 10 min.

   Repeat Step 4 two more times
   (To purify myofibrils after MFIs, start at Step 5)

5. Discard supernatant.
   a. Re-suspend pellet in 6 volumes (v/w) of standard salt solution + 1% Triton X-100 by homogenizing for 10 s.
   b. Centrifuge at 1500 × g for 10 min.
6. Discard supernatant.
   a. Re-suspend pellet in 6 volumes (v/w) of standard salt solution + 1% Triton X-100 by homogenizing for 10 s.
   b. Centrifuge at 1500 × g for 10 min.
7. Discard supernatant.
   a. Re-suspend pellet in 8 volumes (v/w) of standard salt solution by stirring vigorously with polyethylene stirring rod.
   b. Centrifuge at 1500 × g for 10 min.
8. Discard supernatant.
   a. Repeat Step 7 two times, but suspend in 8 volumes (v/w) of 100 mM KCl instead of standard salt solution.
   b. Centrifuge at 1500 × g after each suspension.
9. Discard supernatant.
   a. Re-suspend pellet in 8 volumes (v/w) of 100 mM KCl by homogenizing 3 s.
   b. Centrifuge at 1500 × g for 10 min.
10. Discard supernatant.
    a. Re-suspend pellet in 8 volumes (v/w) of 100 mM NaCl by homogenizing for 3 s.
    b. Centrifuge at 1500 × g for 10 min.
11. Discard supernatant.
    a. Re-suspend pellet in 8–10 mL of 100 mM NaCl homogenizing for 3 s.
    b. Prepare a slide for microscope to check for a protein.
    c. Do a protein analysis of suspension.

RESULT: Purified myofibrils free of membranes.
Myofibril purification solutions:

Standard Salt Solution	**1 L**	**2 L**	
100 mM KCL	7.46 g	14.92 g	
20 mM K-Phosphate:			
KH_2PO_4	1.36 g	2.72 g	
$K_2H_2PO_4$	1.75 g	3.50 g	
2 mM $MgCl_2$ (MW 95.21)	0.19 g	0.38 g	
2 mM EGTA	0.76 g	1.52 g	
1 mM NaN_3 (pH 6.8)	0.065 g	0.13 g	
Standard Salt Solution + 1% Triton X-100	**500 mL**	**1 L**	**2 L**
Standard Salt Solution	495 mL	990 mL	1980 mL
Triton X-100 (weighed on a balance)	5 g	10 g	20 g
100 mM KCl	**1 L**	**2 L**	
KCl	7.46 g	14.92 g	
100 mM NaCl	**500 mL**	**1 L**	
NaCl	2.92 g	5.84 g	

***** Make 100 mL of 1M NaN_3 and then add 1 mL/L to get 1 mM *****

Heat-Labile Collagen Analysis

Chemicals needed:	
NaCl	Sodium acetate trihydrate
$CaCl_2$	Isopropanol
KCl	Chloramines T
dd H_2O	4-dimethylaminobenzaldehyde
H_2SO_4	Perchloric acid
Citric acid monohydrate	Hydroxyproline
NaOH	HCl
Ringer's Stock Solution	0.25 Ringer's Solution:
7.0 g NaCl	250 mL Ringer's Stock
0.026 g $CaCl_2$	Bring to volume in 1 L volumetric flask with dd H_2O
0.35 g KCl	

Dissolve ingredients in 1 L dd H_2O

7N sulfuric acid:

375 mL H_2SO_4
750 mL dd H_2O

Add 750 mL dd H_2O to a 2 L volumetric flask. Place this in an ice bucket under the hood and pull the sash down to protect your eyes. Slowly with agitation, add the H_2SO_4. Dilute to volume with dd H_2O and cool to room temperature.

Buffer solution:

30.0 g citric acid monohydrate
15.0 g NaOH
90.0 g sodium acetate trihydrate
290 mL isopropanol

Dissolve the first three (3) ingredients in 500 mL dd H_2O. Put them in a 1 L volumetric flask, add the isopropanol. Adjust the pH to 6.0 with NaOH or acetic acid. Dilute to volume with dd H_2O.

Note: Solution is stable for 2 mo in a DARK bottle at 4°C.

60% perchloric acid:

428.33 mL 70% perchloric acid
50 mL dd H_2O

Add 50 mL dd H_2O. When cool, bring to 500 mL total volume with dd H_2O.

Note: Can double this to make 1 L.

Hydroxyproline stock solution:

0.025 g hydroxyproline
250 mL 0.001 N HCl

0.001N HCl:

250 μL HCl
249.75 mL dd H_2O

Oxidant solution:
1.41 g chloramines T
Dissolve in 100 mL of buffer solution.
Note: Stable for one wk at 4°C.
Note: Oxidant solution and color reagent are often made in double batches.

Color reagent:

10 g 4-dimethylaminobenzaldehyde (DMABA)
35 mL 60% perchloric acid
65 mL isopropanol

Dissolve DMABA in perchloric acid. Slowly, under agitation, add 65 mL isopropanol.
Note: Stable for only 24 h in a dark bottle.
Heat-Labile Collagen Analysis
Collagen: Determination of Hydroxyproline

Centrifugation:

Prepare the sample

1. Set the water bath to 78°C. While it is heating, weigh out samples (each should be 4.0 and 4.1 g), and record the weight. Each sample should be done in duplicate.
2. Add 12 mL of 0.25 Ringer's solution in each tube. Stir each tube 20–30 times with a separate glass rod for each tube.
3. Place the tubes in the water bath at 78°C for 60 minutes. Stir tubes every 5 min 10–15 revolutions.
4. Places tubes in cooler for 15 min. The procedure can stop here for the evening if the tubes are capped and left in the cooler no longer than overnight. It is preferable to centrifuge the tubes immediately after they cool.

Centrifuge

1. Prepare the centrifuge with the proper rotor and make sure the temperature is set to 2°C.
2. Place the tubes in the centrifuge. Make sure it is balanced and the lid is screwed down tightly. Centrifuge at 15,000 rpm for 20 min at 2°C.
3. While samples are in the centrifuge, label cooking tubes. There will be a tube for the supernatant ("S") and one for the residue ("R") of each sample.

4. Remove tubes from the centrifuge once it has stopped and decant the supernatant into the "S" tube. Do not allow any meat to get into the tube.
5. Add 8 mL of 0.25 Ringer's solution to the meat pellet in each centrifuge tube. Using a glass rod stir each tube 10–15 × .
6. Centrifuge again at 15,000 rpm for 20 min at 2°C.
7. Again pour the supernatant into the "S" tube (adding to the supernatant poured off after the first centrifugation).
8. Add another 5 mL of dd H_2O to each tube, mix well, and pour this residue into the "R" tube. Rinse the rube with another 5 mL of dd H_2O and pour this into the same "R" tube and replace the lids.
9. If so desired, the procedure can stop for the night at this point. If so, place tubes ("S" and "R") in the cooler.

Cooking:

1. Heat oven inside the fume hood up to 105°C. Wear gloves and a laboratory coat, and keep hood sash down to protect your face.
2. Add 30 mL of 7N sulfuric acid to each supernatant ("S") tube and add 20 mL to each residue ("R") tube. Be careful not to breathe the fumes of the sulfuric acid; if you do, drink plenty of water afterwards to flush it from your kidneys.
3. Screw the lids onto the tubes and place them in the oven. Keep the hood on at all times, let samples cook for 16 h.
4. After the samples have cooked for 16 h, take them out of the oven with tongs, "rubber hands" or insulated gloves. Take the lids off the tubes immediately to prevent the formation of a vacuum. If this happens, wait until the sample has cooled and break off the lid. Allow the samples to sit under the hood with their lids off while they cool and the acid vapors escape.
5. Once the tubes have cooled enough to handle, transfer the hydrolysate into volumetric flasks. The content of the "S" tubes go into 200 mL flasks, and the contents of the "R" tubes go into 500 mL flask. Rinse the cooking tubes with water and pour into the flasks; then bring the flasks up to their respective volumes.
6. Filter the diluted samples with no. 2 filter paper into the labeled collection flasks (disposable plastic centrifuge tubes with caps), collecting at least 50 mL. The remainder of the liquid in the volumetric flask is to be discarded.
7. Cap the filtered samples and store them in the cooler. The filtrate is stable for 2 wk at 4°C.

Color step:

Make standard solutions for the color curve with the following dilution:

mL hydroxyproline stock solution	mL dd H_2O
2.0	98
4.0	96
6.0	94
8.0	92
10.0	90

1. Add 2 mL of each standard solution to the duplicate test tube (two tubes per dilution). Make two blank tubes with 2 mL dd H_2O each. Preheat the water bath to 60°C at this time.
2. Pipette 2 mL of each filtrate into the duplicate test tube (two tubes per "R" or "S"; four tubes per original sample).
3. To all tubes (including standards and blanks) add 1.0 mL of oxidant solution.
4. Vortex the tubes, then let them sit for exactly 20 min at room temperature.
5. After the tubes have sat for 20 min, add 1.0 mL of the color reagent to each tube and vortex.
6. Place the tubes in the heated water bath for exactly 15 min, placing a sheet of aluminum foil over the top of all the tubes before closing the lid.
7. Cool the tubes to room temperature, then transfer the samples to cuvettes. Read on the spectrophotometer at 558 nm. The reagent is light-sensitive, so the sooner the samples can be read and the less light they receive, the better.

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7 Water-Holding Capacity of Meat

Jason K. Apple and Janeal W.S. Yancey

Introduction

Water comprises approximately 75% of the weight of meat (Table 7.1), and the ability of meat and meat products to retain water varies considerably. For example, it has been estimated that as much as half of all the pork produced in the United States has excessive moisture losses (Kauffman et al., 1992; Stetzer and McKeith, 2003). Unacceptable moisture losses can cause reductions in carcass and primal weights during handling and transportation (Kauffman et al., 1978), product yields after further processing (Jeremiah and Wilson, 1987; Kauffman et al., 1992), and sensory attributes of cooked meat products (Oeckel et al., 1999). Myoglobin, the primary pigment for meat color, is water-soluble; thus, excessive water loss leads to meat color fading. Consumers discriminate against the faded meat color (Morgan et al., 1994; Brewer et al., 1998) and excessive, unsightly purge that develops in, and sometimes leaks out onto, packages of meat with low water retention (Person et al., 2005; Otto et al., 2007).

Reiner Hamm (1960) defined *water-holding capacity* (WHC) as the ability of meat to retain its inherent water during force application and/or processing (i.e., cutting, pressing, grinding, packaging, curing, thermal processing, etc.). When fresh meat is cut, the pinkish-red solution that contains water-soluble sacroplasmic proteins, lactic acid, and myoglobin (Savage et al., 1990) and which oozes from the cut surface, is referred to as *purge* (also termed "exudate," "drip," or "weep"). Losses of water (or purge) from meat can occur via evaporation, gravitational drip, thawing, or cooking, and low WHC (excessive moisture loss) obviously reduces the weight of saleable fresh meat products (Offer and Knight, 1988a; Melody et al., 2004). In the pork industry, for instance, low WHC results in losses of $26.2 million ($0.41/pig slaughtered in Germany; Fischer, 2007) to $70 million ($0.50/pig slaughtered in the United States; Morgan et al., 1994; Stetzer and McKeith, 2003) annually. Thus, WHC is debatably one of the most important meat quality attributes today, especially for the case-ready and further-processed meats industries.

Water is a dipolar molecule that is attracted to charged molecules (like myofibrillar and stromal proteins) and polar molecules without charges, and is found compartmentalized into distinct areas in muscle. A very small fraction of water (about 0.3–0.5%) is located bound within the myofibrillar proteins, and is referred to as *constitutional water* (Hamm, 1986). Among the amino acids of the myofibrillar proteins, glutamic acid and lysine have charged side groups to which water is strongly attracted and bond; whereas glutamine and tyrosine contain nitrogen and oxygen atoms in side groups that have sufficient polarity, due to concentrations of electrons, to attract and bind

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Table 7.1 Total moisture content (%) of select muscles

Muscle	Beef [a]	Beef [b]	Pork [c]
Adductor	72.28	72.86	76.21
Biceps femoris	71.22	71.61	75.15
Complexus	–	72.02	–
Gluteus medius	71.85	71.44	75.25
Infraspinatus	70.50	70.81	75.78
Latissimus dorsi	–	72.34	71.30
Longissimus thoracis et lumborum	69.95	72.34	71.30
Pectoralis profundi	72.05	73.32	73.96
Psoas major	69.25	70.52	74.76
Rectus femoris	72.55	73.33	77.28
Semimembranosus	71.97	72.79	75.06
Semitendinosus	72.90	73.27	74.98
Serratus ventralis	67.08	68.77	73.31
Supraspinatus	72.86	74.29	75.99
Tensor fasciae latae	–	74.08	73.47
Teres major	–	73.54	–
Triceps brachii	72.56	73.23	75.75
Vastus intermedius	–	72.91	76.33
Vastus lateralis	–	73.54	–
Vastus medialis	–	75.02	76.64

[a] Brackebusch et al. (1991)
[b] Von Seggern et al. (2005).
[c] Porcine Myology (www.porcine.unl.edu).

water (Wismer Pedersen, 1971). Conversely, side groups of leucine, alanine, and valine (comprised solely of carbon and hydrogen atoms) are nonpolar (hydrophobic), which, being electrically neutral, actually repel water.

By definition, *bound water* is water that is held tightly by myofibrillar proteins, does not move among water compartments, is resistant to freezing, and can only be removed by severe drying processes not including conventional cooking (Fennema, 1996). Yet, there is about 0.5 g of water per gram of protein (Kuntz and Kaufmann, 1974), so bound water represents only about 4–5% of water in muscle (Hamm, 1960; Cooke and Wein, 1971).

The largest portion of water is found held within the structure of each myofibril (Eisenberg and Kuda, 1975), but not bound to myofibrillar proteins, and is commonly referred to as *immobilized water*. Immobilized water is held firmly within the myofibril by either steric (space) effects and/or by attraction to the bound water, but is not bonded with the myofibrillar proteins. Although immobilized water is not very mobile among water compartments immediately after the completion of rigor mortis, it can be removed by conventional heating and converted into ice during freezing (Fennema, 1996). *Free water* flows from muscle tissue unimpeded and is held within muscles by very weak capillary forces. The last form of water is termed *extracellular water*, and represents the water that escapes the muscle cell structure as purge sometime after the rigor process.

Postmortem Muscle Metabolism and the WHC of Meat

The cessation of blood circulation at death severely restricts oxygen availability to muscles, thereby shifting muscle metabolism from aerobic metabolism of available lipids and circulating

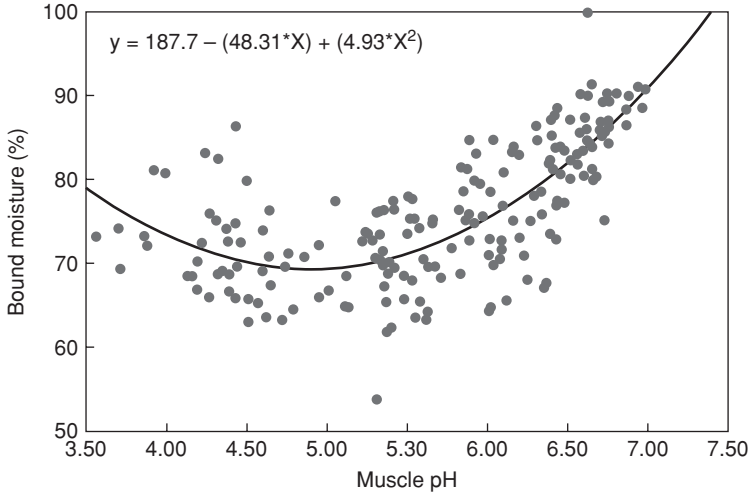

Figure 7.1 The effect of altering the muscle pH of beef longissimus muscle with lactic acid on the percentage of bound water. (Adapted from Sawyer et al., 2008, 2009).

carbohydrates to anaerobic metabolism of muscle glycogen reserves. In addition, the end-product of postmortem glycogenolysis is lactic acid, and the accumulation of lactic acid is responsible for the curvilinear decline in postmortem muscle pH from approximately 7.08 to 7.3 (Bate-Smith, 1948; Sahlin, 1978) to an ultimate pH value of 5.4–5.7 (Greaser, 1986). When postmortem muscle pH reaches the *isoelectric point* (*pI*) of the major myofibrillar proteins (pI ≈ 5.0–5.3; Wismer Pedersen, 1971; Hamm, 1986), the net charge of contractile proteins is zero (Fig. 7.1). The virtually equal number of positive and negative charges of the contractile proteins at the pI causes the charged portions of the proteins to be attracted to each other, thereby reducing the amount of water that can be attracted to the myofibrillar proteins. However, when the pH is below the pI, there is a surplus of positive charges, and when the pH is above the pI, there is an excess of negative charges, with the result being greater attraction to water (Fig. 7.2). WHC increases and decreases linearly as pH increases (Fig. 7.3) or decreases.

The electrostatic forces that maintain the spacing between myofilaments are also reduced at the pI (Rome, 1967, 1968). When the normal repulsion between myofilaments is eliminated at the pI, the interfilamental spacing between thick and thin filaments is reduced by the formation of rigor bonds, resulting in less space within the myofibril for water (Offer and Knight, 1998b). Therefore, water moves from the myofibril into the extramyofibrillar spaces, where it eventually is lost from the muscle cell (Offer and Knight, 1998b; Offer, 1991; Offer and Cousins, 1992).

Drip Channels and Postmortem Aging

The myofilament lattice spacing does not change much during the first few hours postmortem (Fig. 7.4), but there is a rapid reduction in the lattice spacing with the onset of rigor mortis (Offer et al., 1989). This decrease in lattice spacing is partially due to the development of permanent actomyosin bonds (Offer and Knight, 1998b). As the myofilaments and myofibrils shrink during the rigor process, water begins to accumulate around the perimyseal network of muscle bundles, and then

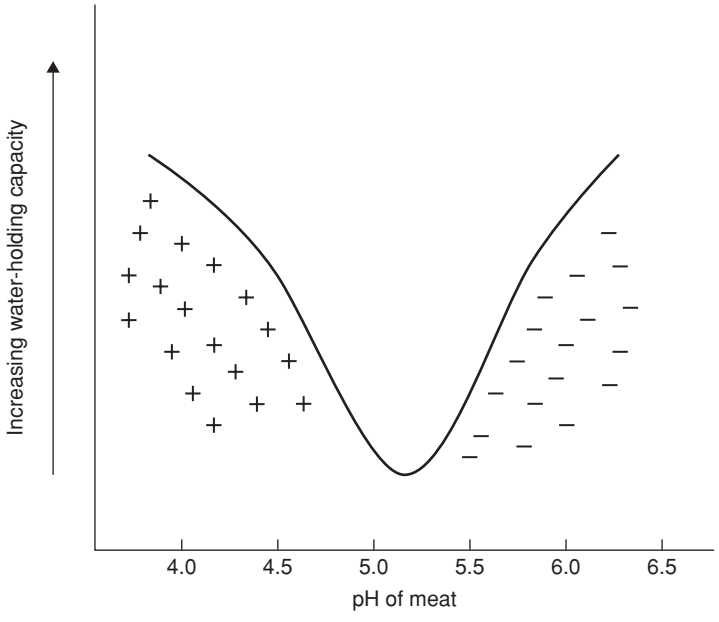

Figure 7.2 The effect of muscle pH on the immobilized water in meat. When muscle pH approaches the isoelectric point (pI) of 5.0–5.3, the net charge of myofibrillar proteins is equal. Yet, the attraction to the positive charge of water increases as the pH declines below pI, whereas the attraction to the negative charge of water increases as the pH increases above the pI. (Adapted from Wismer Pedersen, 1971).

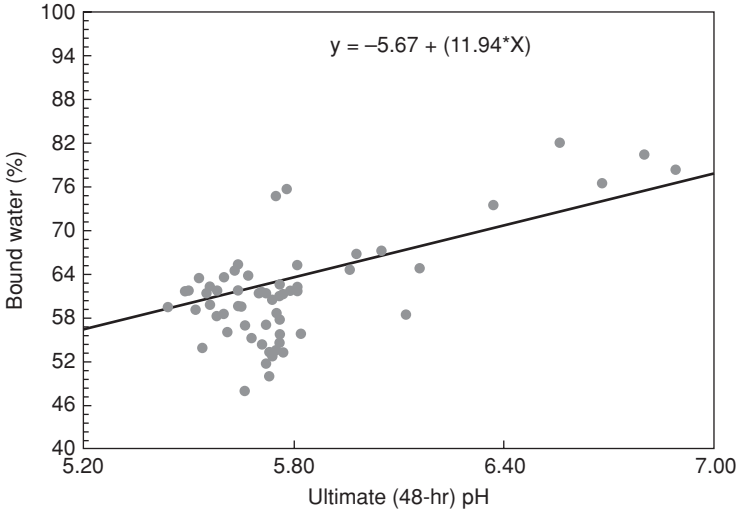

Figure 7.3 The effect of increasing muscle pH on the percentage of bound water in the longissimus muscle from beef carcasses ($R^2 = 0.552$; J.K. Apple, unpublished results).

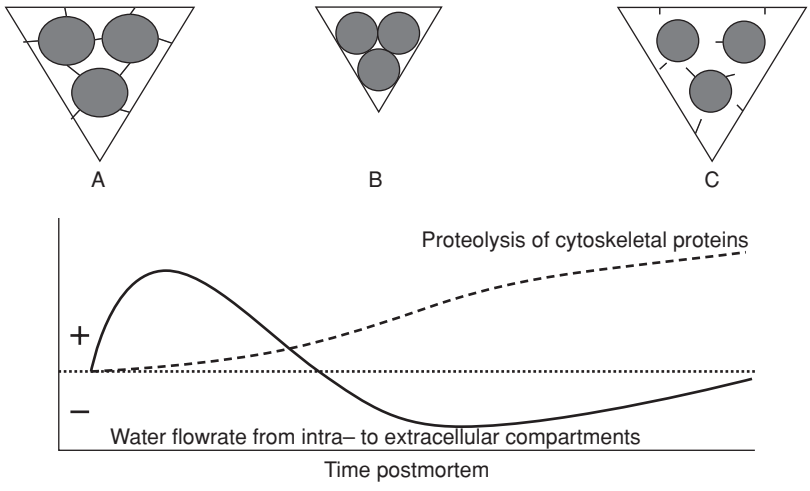

Figure 7.4 Relationship between time postmortem, quantity of proteolysis of cytoskeletal proteins, and water flux rate, where: (a) a pre-rigor muscle cell with three myofibrils connected to each other and to the cell membrane by the cytoskeleton; (b) shrinkage of individual myofibrils leads to shrinkage of the whole muscle cell causing the movement of water out of the muscle cell into the extracellular space; and (c) proteolysis of cytoskeletal proteins removes the restrictions of cytoskeleton on myofibrils causing the movement of water from the extracellular space into the muscle cell (Kristensen and Purslow, 2001).

the endomyseal network of individual muscle cells, forming distinct, extracellular water channels between muscle cells and bundles of cells (Offer and Knight, 1998b; Morrison et al., 1998, 2000). Moreover, in cases of severe muscle contraction, the thin and thick filaments are even closer together at the onset of rigor mortis; thus, even more water can be expelled from the myofibril (Offer and Trinick, 1983; Honikel and Kim, 1986), which explains the negative correlation between sarcomere length and drip loss (Fig. 7.5) (Honikel et al., 1986).

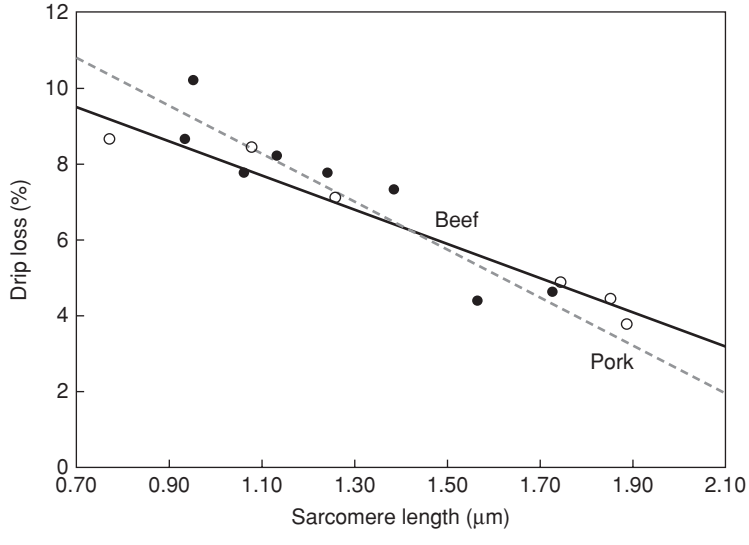

Figure 7.5 The relationship between sarcomere length and drip loss from beef (○) and pork (●) after 7 days of refrigerated storage (Honikel et al., 1986).

Interestingly, several researchers have shown that the WHC of meat increases with postmortem aging (Davis et al., 2004; Straadt et al., 2007), and a growing body of evidence suggests that postmortem proteolysis may be responsible for the improved WHC of aged meats (Morrison et al., 1998; Huff-Lonergan and Lonergan, 2005). Taylor et al. (1995) observed that drip loss was reduced when the extracellular matrix was separated from the cytoskeleton. It was not long before researchers demonstrated that degradation of the cytoskeletal protein desmin by the calpains (Melody et al., 2004; Gardner et al., 2005, 2006; Bee et al., 2007) released the within-cell constraints limiting the available space for water expelled from the myofibril during the onset of rigor mortis (Huff-Lonergan and Longergan, 2007).

Kristensen and Purslow (2001) suggested that postmortem degradation of certain costameric proteins, which attaches myofibrils to the sarcolemma, may also be involved in myofibrillar swelling and accumulation of water forced from the extracellular space. Specifically, the costameric proteins vinculin and talin were extensively degraded in pork with very high WHC (Bee et al., 2007), and degradation of these costameric attachments would reduce the lateral shrinkage transmitted down the entire muscle cell, ultimately increasing the volume within the cell for water accumulation (Huff-Lonergan and Lonergan, 2005). Integrins are proteins involved in the continuous attachment of the cytoskeleton to the extracellular matrix; however, the degradation of integrin may actually have detrimental effects on WHC. Recent research indicates that postmortem integrin degradation leads to the formation of drip channels (Lawson, 2004; Zhang et al., 2006; Straadt et al., 2008), whereas inhibition of the calpains not only inhibits integrin degradation but also blocks the opening of drip channels (Lawson, 2004).

Manipulating the WHC of Meat

Salt

A way to alter the relative number of charged reactive groups on myofibrillar proteins is by the addition of salt. First, sodium chloride affects the osmolarity of the muscle cell and destroys the sarcolemma integrity, which leads to the increased permeability of salt into the muscle fibers and greater fiber hydration (Brewer, 2004). Secondly, it appears that sodium chloride causes a shift in the pI, resulting in enhanced moisture retention and meat fiber swelling (Bendall, 1954; Swift and Ellis, 1956). The reason is that chloride anions bind rather extensively to the positively charged reactive groups of actomyosin, whereas sodium cations are only weakly bound to the negatively charged reactive groups (Hamm, 1960); thus, the pI is moved towards a lower pH (Fig. 7.6). Furthermore, the side chains are no longer attracted to one another, which allows for the widening of the muscle structure, and increased WHC via myofibrillar swelling. In fact, if salts are added to pre-rigor meat, the influx of water into the interfilamental space increases the distance between myofibrils, thereby breaking the actomyosin linkage, leading to additional protein swelling (Brewer, 2004).

The WHC of pre-rigor meat can be increased with increasing amounts of added salt from 1.8% to 2.0% (Fischer et al., 1982). Hamm (1986) explained that the effect of low levels of salt on WHC was irreversible, and was caused by the inhibition of rigor mortis development by a strong repulsion between adjacent proteins caused by a combination of residual ATP content, high muscle pH, and high ionic strength. On the other hand, addition of from 3.0% to 5.0% salt causes decreased swelling of meat proteins, possibly by the displacement of magnesium and potassium by sodium (Brewer, 2004). Interestingly, there is a rapid, muscle protein swelling when 5.0% to 10.0% salt is added to

Figure 7.6 The effect of adding salt (NaCl) on the immobilized water in meat. Chloride ions are bonded to positively charged groups of myofibrillar proteins, whereas sodium ions are weakly bound to the negatively charged reactive groups, and the net effect is the displacement of the isoelectric point (pI) toward a lower pH. (Adapted from Wismer Pedersen, 1971).

meat products, and the increased WHC is a result of an increase in the ionic strength, which causes the dissociation of actomyosin complexes (Brewer, 2004).

Phosphates

In 1952, the federal government allowed the addition of phosphate to cured meat products, such as hams, bacon, and smoked pork loin; yet, in 1982, the addition of phosphate was approved for use in fresh, frozen, and pre-cooked meat products. It is well known that addition of phosphate to meat products increases WHC and muscle tissue swelling, which results in greater retail cut yields, while decreasing drip and cooking losses. Phosphate also enhances the ability of salt to increase myofibril swelling and water retention (Offer and Trinick, 1983; Patterson et al., 1988). Additionally, enhancement of meats with a solution containing water, salt, and less than 0.5% phosphate results in dilution of the protein matrix and improvements in cooked meat juiciness, whereas the physical breakdown of myofibrillar proteins by the solution injection needles leads to improvements in the tenderness of phosphate-enhanced meat products. Other research has also demonstrated that phosphates can decrease microbial growth (Dickson et al., 1994; Kim and Slavik, 1994), reduce oxidative rancidity (Jimenez-Villarreal et al., 2003; Duong et al., 2008), and preserve the color of fresh meats (Pohlman et al., 2002a, 2002b).

The most common forms of phosphate added to meat products are di- or pyrophosphates, which contain two phosphate groups, and tripolyphosphates, which contain three phosphate groups. These structures result in maximum water-binding activity in fresh meat. Mono- or orthophosphates, which consist of only one phosphate group; tetraphosphate, consisting of 4–10 phosphate groups; and hexametaphosphate, which consist of a chain of 10 or more phosphate groups, are not commonly used in meat products. It is believed that phosphates improve the WHC of meat proteins in three ways: (1) increase the ionic strength; (2) increase or decrease muscle pH away from the pI; and (3) impart an “intrinsic phosphate property.”

Myofibrillar proteins are more soluble at higher ionic strengths, and the ionic strength of phosphates decreases as the chain length of phosphates increases. Pyrophosphate and tripolyphosphate, as well as hexametaphosphate, can increase ionic strength, with polyphosphates providing similar increases in ionic strength as sodium chloride (Brewer, 2004). Thus, phosphates increase the ionic strength, protein solubility, and myofibril swelling. Interestingly, when studying the effects of ionic strength and pH on cook yields of restructured beef rolls, Trout and Schmidt (1986) found that cook yields were strongly related to ionic strength at normal meat pH (5.5). However, at higher pH levels, that relationship was less evident.

It has already been established that adjusting the pH of meat away from its pI can increase its WHC, and phosphates are quite efficient at adjusting meat pH. Most phosphates are added in the basic form as a sodium or potassium phosphate with a pH of 7.0–10.0 (Brewer, 2004; Baublits et al., 2006), but acid pyrophosphates, with a pH of approximately 4.0 (Baublits et al., 2005), can lower meat pH.

The ability of phosphates to buffer or alter meat pH is dependent on chain length, with maximal buffering capacity achieved with monophosphate, with buffering capacity decreasing with increasing chain length. It should be noted, however, that meat proteins have a very high buffering capacity; therefore, unless included at relevant quantities, a phosphate-solution with a pH of 10 may shift meat pH only 0.1–0.3 pH units (Brewer, 2004), which would result in minimal improvements in the WHC of meat at or near the pI.

In the live animal, inorganic phosphates such as adenosine triphosphate (ATP) dissociate the actomyosin complex, and phosphates added postmortem act much the same way (Bendall, 1954; Yasui et al., 1964a, 1964b). When actomyosin is dissociated, this allows for expansion of the myofibril and makes more room for free water (Patterson et al., 1988). Thus, inclusion of phosphate in a meat system also works with salt to extract the ends of the A-band in the sarcomere, which also allows for myofibril expansion (Offer and Trinick, 1983; Patterson et al., 1988). Moreover, swelling of the myofibril is inhibited by titin, and titin can be extracted by phosphates, thereby releasing its inhibition on myofibril swelling.

Lastly, some researchers believe that phosphate may allow for increased myofibrillar swelling by its ability to chelate divalent metal ions (Hamm, 1960). Specifically, calcium and magnesium ions react with negatively charged reactive groups on adjacent myofibrils to inhibit actomyosin swelling; however, polyphosphates are capable of chelating these divalent cations bound to actomyosin resulting in the cleavage of actomyosin bonds and increased hydration of myofibrillar proteins (Brewer, 2004). However, Hellendoorn (1962) found that EDTA—a known calcium-chelator—actually reduced the WHC of ground beef; Inklaar (1967) showed that phosphate addition of comminuted meat did not affect the concentration of calcium and magnesium ions; and Irani and Callis (1962) reported that neither salt, pyrophosphate, nor tripolyphosphate chelated calcium or magnesium at pH values less than 7.0. Furthermore, Offer and Trinick (1983) reported that myofibrils swelled when bathed in sodium chloride and pyrophosphate regardless of the presence of magnesium chloride.

Acid Marination

Meat naturally exists at a pH above its pI (Hamm, 1960), so there are slightly more negatively charged than positively charged side-groups in the protein matrix. Yet, when acid is continually added and pH is lowered, negatively charged side-groups are gradually protonated, resulting in an increase in the number of positively charged groups that begin to repel one another, making greater

intracellular space for water (Gault, 1991; Hamm, 1960). Furthermore, water is attracted to these positively charged reactive groups, and swelling of muscle fibers reaches a maximum at pH 3.5 (Gault, 1991), where it is assumed that all available carboxyl groups in the protein are protonated. Greater addition of positively charged protons (below pH 3.5) induces an increased binding of anions, which screens the positive charges on the protein and decreases the repulsion within the protein (Gault, 1991).

Even though pH was lowered by acidic marination, Gault (1985) observed little swelling in beef muscle between the ultimate pH of the non-marinated muscle and pH 4.5; however, at pH values below 4.5, swelling dramatically increased until pH reached 3.4 for *longissimus* muscle and *triceps brachii* and pH 3.2 for the *infraspinatus.* When the marinated samples were cooked, however, all lost weight, but those with a final pH below 4.3 retained a substantially greater amount of water (Gault, 1985).

Several researchers have noted increases in the hydration of the collagen–gelatin matrix of muscle (Rao and Gault, 1989; Rao et al., 1989; Aktaş and Kaya, 2001), but to a much lesser extent than that of muscle tissue, especially above pH 4.3 (Gault, 1991). Below pH 4.3, when the muscle fibers have reached maximum swelling capacity, the endomysium and perimysium (composed of primarily collagen) will continue to swell to a pH value of approximately 2.5 (Rao and Gault, 1989; Rao et al., 1989).

Factors Influencing the WHC of Meat

Several factors have been repeatedly shown to impact the WHC of meat. Genetics and preslaughter animal management have received the greatest amount of research, but animal nutrition, electrical-stimulation, and carcass chilling can also affect the WHC of meat and meat products.

Genetics

Two major genes have been identified that have dramatic effects on the WHC of pork: (1) the *halothane* (HAL) *gene* associated with the porcine stress syndrome, and (2) the *rendement Napole* (RN) gene associated with "acid" pork. Topel et al. (1968) characterized the porcine stress syndrome as a genetic abnormality, and Eikelenboom and Minkema (1974) use a halothane-screening test to classify pigs as either positive (stress-susceptible) or negative (stress-resistant). Once Mabry et al. (1981) elucidated the inheritance of the halothane gene, it was possible to segregate pigs into three possible halothane genotypes: halothane-negative (NN), halothane-carrier (Nn), and halothane-reactor/positive (nn) pigs. However, it was not until 1991 that a gene probe (HAL 1843®) was developed by Fujii and colleagues (1991) that enabled scientists to accurately distinguish the homozygous-negative and heterozygous-carriers of the halothane gene.

The halothane gene (RYR1) is located on chromosome 6 and comprises two alleles (Hal^N = normal allele and Hal^n = recessive mutant allele). Effects of the halothane gene are due to a mutation at position 1843 of the gene that encodes for the ryanodine receptor (Fujii et al., 1991; Otsu et al., 1991) of the sarcoplasmic reticulum calcium-release channel membrane protein. The ryanodine-receptor protein is located on the surface membrane of the sarcoplasmic reticulum and determines the rate of calcium release and uptake during the initiation of muscle contraction and subsequent relaxation. When halothane-carrier or reactor pigs are exposed to a stressor, calcium concentrations are elevated in muscle resulting from reduced release-rate and/or inability of the

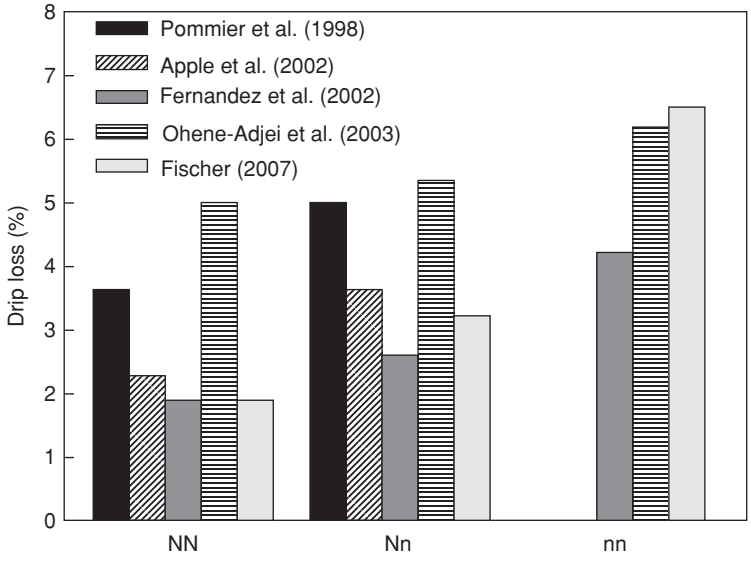

Figure 7.7 Effect of the halothane genotype on moisture retention of the longissimus muscle from pigs.

sarcoplasmic reticulum to sequester calcium after a muscle contraction. Furthermore, increased muscle calcium levels lead to elevated energy metabolism and, during the conversion of muscle to meat, this rapid postmortem metabolism while carcass temperature is high (>36°C) results in partial denaturation of skeletal muscle proteins and elevated moisture losses (Fig. 7.7).

The rendement Napole gene was first discovered in Hampshire pigs (Monin and Sellier, 1985), and is characterized by a 70% increase in muscle glycogen stores (Monin et al., 1992; Estrade et al., 1993b). Moreover, pigs can be classified into two genotypic groups—homozygous normal (rn + /rn +) and RN carriers (RN−/rn + and RN−/RN−)—based on the bimodal distribution of muscle glycogen content (Monin and Sellier, 1985; Fernandez et al., 1992b; Le Roy et al., 1990). It was discovered in 2000 that an autosomal dominant mutation of the protein kinase AMP-activated γ3 subunit gene (PRKAG3), which encodes the γ3 isoform of AMP-activated protein kinase, was the causative gene for the RN- phenotype (Milan et al., 2000). Additionally, Milan and colleagues (2000) found that the mutation was a substitution of arginine by glutamine at the codon 200 of chromosome 15; yet, Ciobanu et al. (2001) discovered that the substitution of isoleucine by valine at codon 199, along with the mutation at codon 200, was responsible for the abnormal glycogen accumulation.

These abnormally high muscle glycogen reserves lead to excessive intramuscular lactic acid accumulation postmortem and pH values approaching the pI (Fig. 7.8), which elicits dramatic reductions in WHC and elevated moisture losses (Fig. 7.9) from meat (Lundström et al., 1996, 1997; Lebret et al., 1999; Hamilton et al., 2000). Because water, in muscle, is bound to both glycogen and protein: (1) elevated muscle glycogen levels would bind more water, and, during postmortem metabolism of glycogen, WHC of the muscle would be severely decreased (Lebret et al., 1999); and (2) several researchers have shown a reduction in total protein (Estrade et al., 1993a; Lundström et al., 1996) and/or denaturation of myosin tails and sarcoplasmic proteins in RN^- pigs (Deng et al., 2002), which would also result in greater drip losses.

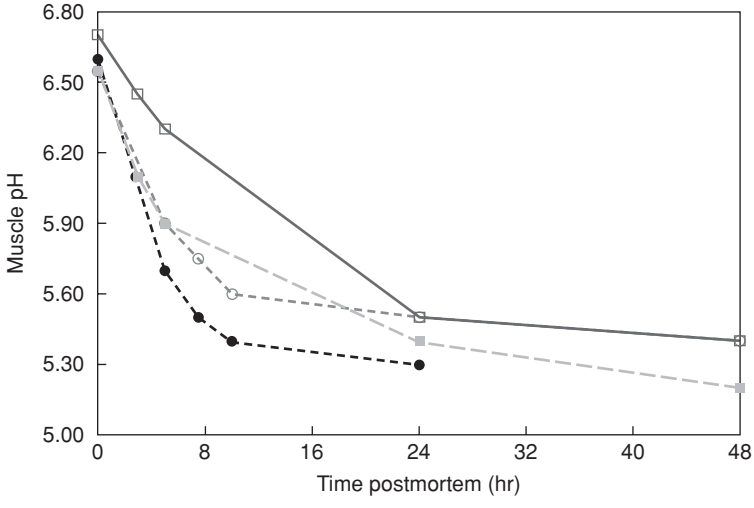

Figure 7.8 Effects of the rendement Napole genotype on pH decline in the longissimus muscle (LM). Open circles (○) and blocks (□) represent the pH decline in the LM from normal (rn + /rn +) pigs, whereas closed circles (●) and blocks (■) represent the pH decline in the LM from carrier (RN-) pigs (○ and ●—adapted from Josell et al., 2003; □ and ■—adapted from Lindahl et al., 2006).

Within the past ten years, mapping of the porcine genome has elucidated a number of quantitative trait loci (QTL) involved in pork quality, and QTL specific for WHC were discovered on chromosomes 1, 2, 4, 5, 6, 9, 11, 13, 14, 15, and 18 (Bertram et al., 2000; De Koning et al., 2001; Malek et al., 2001; Su et al., 2004; Thomsen et al., 1998). Once a QTL has been identified and mapped, the underlying genes involved with WHC can then be identified (Table 7.2). Moreover, there are a number of other candidate genes that have been shown to impact muscle pH, which would also

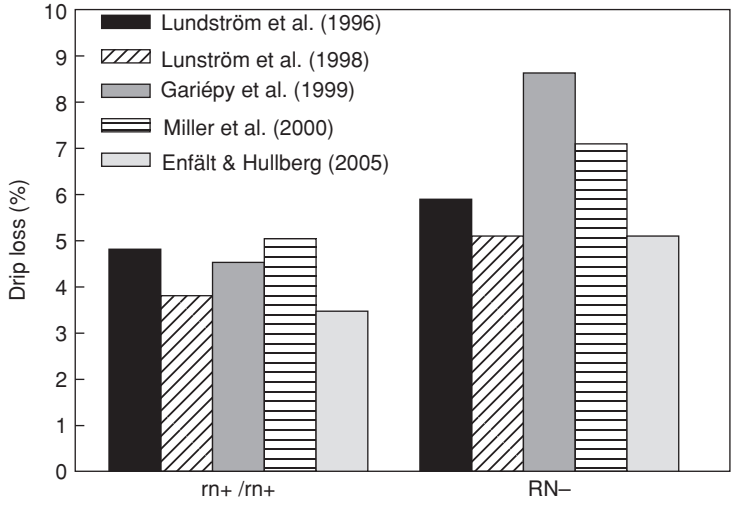

Figure 7.9 Effect of the rendement Napole genotype (rn + /rn + = normal and RN− = carriers) on moisture retention of the longissimus muscle from pigs.

Table 7.2 A sample of candidate genes mapped on porcine chromosomes containing Quantitative Trait Loci (QTL) regions for pork drip loss/water-holding capacity (Jennen et al., 2007)

Chromosome	Gene	Gene description	Reference
2	ACTN3	Actinin α-3	Davoli et al. (2002)
	FTH1	Ferritin, heavy polypeptide 1	Ponsuksili et al. (2002)
	MYOD1	Myogenic differentiation 1	Cepica et al. (1999)
	TTID	Titin immunoglobulin domain protein (myotilin)	Davoli et al. (2002)
3	ACTB	Beta actin	Thomsen et al. (1998)
	ATP2A1	ATPase Ca^{2+}-transporting, fast-twitch 1	Ciobanu et al. (2001)
	HUMMLC2B	Myosine regulatory light chain 2	Davoli et al. (2003)
	PHKG	Phosphorylase kinase γ-subunit	Looft et al. (1999)
5	MYBPC1	Myosin-binding protein C	Wu et al. (2004)
	WYF5	Myogenic factor 5	Cepica et al. (1999)
	MYF6	Myogenic factor 6	Vykoukalova et al. (2003)
	PPP1R12A	Protein phosphatase 1 regulatory subunit 12A	Davoli et al. (2002)
	PFKM	Phosphofructokinase	Fontanesi et al. (2003)
	PRKAG1	Protein kinase, AMP-activated, γ_1 subunit (non-catalytic)	Haberkern et al. (2004)
18	CAPZA2	Capping protein (actin filament)muscle Z-line, α_2	Campbell et al. (2001)
	IFRD1	Interferon-related development regulator 1	Davoli et al. (2002)
	PGAM2	Phosphoglycerate mutase 2	Davoli et al. (2000)
	PRKAG2	Protein kinase, AMP-activated, γ_2 subunit (non-catalytic)	Haberkern et al. (2004)

Candidate genes were selected based on function during muscle development and metabolism, as well as their association with drip loss.

alter the WHC of meat and meat products (Briley et al., 1996; Davoli et al., 2000, 2002; Fontanesi et al., 2003). Even though a number of genes have been identified that are directly and indirectly associated with the WHC of meat, the gene interactions are largely unknown at this time (Jennen et al., 2007).

Preslaughter Animal Management

Most livestock producers withhold feed from animals 8 to 16 hours before loading and transportation to reduce gut fill and shrinkage, mortality during transportation, and the risk of microbiological cross-contamination of hides or skin. Moreover, feed withdrawal for at least 24 hours before slaughter has been shown to reduce antemortem muscle glycogen reserves in pigs, leading to elevated early-postmortem pH values and improved pork WHC (Warriss, 1982; Eikelenboom et al., 1991; Wittmann et al., 1994). On the other hand, fasting cattle for 72 hours before slaughter may reduce muscle and liver glycogen levels but had no effect the WHC of beef (Carr et al., 1973).

Negative handling of pigs prior to slaughter can lead to decreased WHC and an increase in the incidence of pale, soft, and exudative (PSE) pork (D'Souza et al., 1998b), whereas negative handling in cattle leads to increased WHC and an increased incidence in dark, firm, and dry (DFD) beef (Wythes et al., 1985, 1988; Jones et al., 1988). Along with injuries associated with fighting when unfamiliar animals are mixed, comingling can cause excessive antemortem glycogenolysis and curtail postmortem muscle metabolism and pH decline, resulting in increased WHC of pork (Warriss and Brown, 1985; Faucitano, 1998; Warriss et al., 1998a) and beef (McVeigh and Tarrant, 1983; Schaefer et al., 1990).

Just about any short-duration stressor imposed immediately prior to the slaughter process can cause excessive glycogen usage in both cattle and hogs and increased variation in meat quality; however, stress in pigs immediately before slaughter can cause body temperature to increase, as well as the lowering of muscle pH values prior to (Enfält et al., 1993; Henckel et al., 2002), or immediately after (Gariépy et al., 1989; van der Wal et al., 1997; Brown et al., 1998; van der Wal et al., 1999; Henckel et al., 2000), stunning—even in pigs free of the halothane genotype (Schäfer et al., 2002)—which leads to reductions in the pork WHC and increased development of PSE carcasses (Hambrecht et al., 2005). Today, most slaughter pigs arriving at a packing plant are afforded, at the least, a 2- to 3-hour lairage period to calm down and recover from any preslaughter stress (van der Wal et al., 1997; Milligan et al., 1998; Warriss et al., 1998b). Yet, Hambrecht and colleagues (2004b) found that lairage durations of 30 minutes to 3 hours were insufficient to promote glycogen replenishment and alter the preslaughter stress effect on pork WHC, whereas extended lairage times (greater than 12 hours) are conducive to fighting and restless activity, which can lead to bruising, additional muscle glycogen usage, and development of DFD pork (Murray et al., 2001).

Nutrition

Even though Goodband et al. (1990) reported improvements in the WHC of the longissimus muscle and semimembranosus as the dietary lysine content increased from 0.6% to 1.0% in swine finisher diets, most research has failed to detect an effect of crude protein or amino acid content (Essén-Gustavsson et al., 1994; Goerl et al., 1995; Unruh et al., 1996; Bidner et al., 2004), energy intake (Lebret et al., 2001; Lee et al., 2002), or energy source (Camp et al., 2003; Carr et al., 2005b; Nuernberg et al., 2005; Teye et al., 2006) on the WHC of pork. Early research demonstrated that feeding pigs sugar prior to slaughter increased muscle glycogen levels, which caused ultimate pH values to be elevated (Briskey et al., 1959, 1960; Fernandes et al., 1979), but WHC was either not affected (Fernandez et al., 1992a), or was actually reduced (Camp et al., 2003). Moreover, preslaughter muscle glycogen reserves were effectively reduced, whereas muscle pH and WHC were increased, by feeding pigs a low-starch and high-fat finishing diet (Rosenvold et al., 2001, 2002, 2003). Henckel and colleagues (2002) concluded that muscle glycogen levels had to be less than 53 μmol/g of wet tissue weight immediately before slaughter to realize the beneficial effects of feeding these low-starch diets on pork WHC. Although other researchers (Bee, 2002; Leheska et al., 2002; Bee et al., 2006) failed to reduce muscle glycogen levels below this threshold or observe differences in WHC, there is compelling evidence to suggest that feeding low-starch diets for the last 21–28 days before slaughter may improve the water-retaining properties of pork.

Supplementing diets with vitamin E (α-tocopherol) at supranutritional levels retards lipid and myoglobin oxidation (Faustman et al., 1989). Furthermore, incorporating 100–200 (Asghar et al., 1991; Lauridsen et al., 1999), 200 (Onibi et al., 2000), 500 (Cheah et al., 1995; Onibi et al., 2000), or 1,000 IU of α-tocopherol (Cheah et al., 1995) in swine diets has been shown to improve the WHC of pork (Fig. 7.10).

In addition, when results from 13 studies were subjected to meta-analysis, Apple et al. (2007) found the consensus of available research was that supplementing swine diets with magnesium effectively improved the WHC of pork (Fig. 7.11). More specifically, WHC was improved by short-duration (one to seven days before slaughter) magnesium-supplementation (D'Souza et al., 1998c, 1999; Hamilton et al., 2002, 2003), but neither long-duration supplementation (Apple et al., 2000,

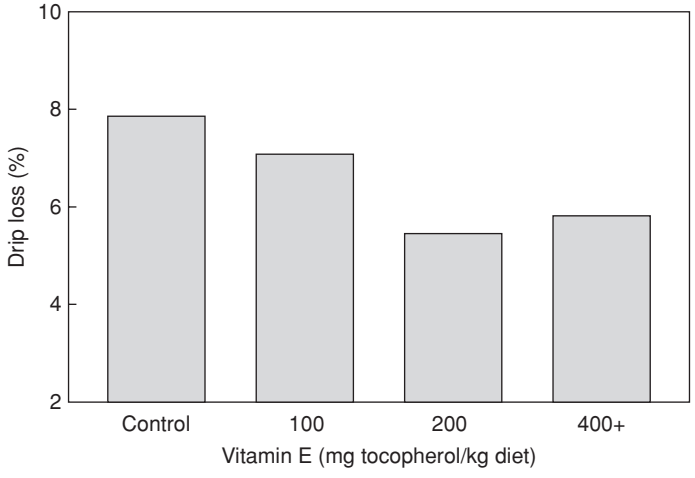

Figure 7.10 The effects of vitamin E (α-tocopherol) supplementation on drip loss from fresh pork. Meta-analysis of the available literature suggested supplementing swine finishing diets with 100, 200, and 400 + mg α-tocopherol/kg of diet reduced drip loss percentages by 10.1, 30.5, and 25.9%, respectively (Apple, 2007).

2002a, 2002b) nor short-duration water treatment with magnesium (Fredrick et al., 2004, 2006) augmented pork longissimus muscle WHC.

The exogenous treatment of pigs with somatotropin or the dietary inclusion of ractopamine hydrochloride (a phenethanolamine β-adrenergic agonist) has repeatedly been shown to improve rate of body weight gain and feed conversion efficiency, as well as increased carcass muscling with the simultaneous reduction in carcass fatness. However, the WHC of the longissimus, semimembranosus, and semitendinosus muscles was not affected by daily injections of porcine somatotropin (McPhee et al., 1991; Goodband et al., 1993; Aalhus et al., 1997; Dugan et al., 1997). Moreover,

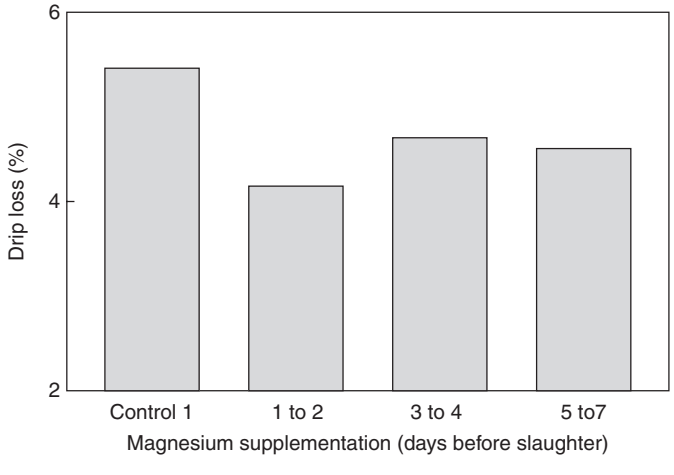

Figure 7.11 The effects of magnesium supplementation on drip loss from fresh pork. Meta-analysis of the available literature indicated reductions in drip losses of 23.1, 13.7, and 15.9% when swine finishing diets were supplemented with magnesium for 1–2, 3–4, and 4–7 days before slaughter, respectively (Apple, 2007).

Uttaro et al. (1993) and Carr et al. (2005a) observed reductions in drip loss from the longissimus muscle of pigs fed 20 mg/kg of ractopamine; however, consensus of the published research indicates that feeding ractopamine does not affect pork WHC (Apple, 2007).

Carcass/Meat Chilling

The rapid decrease in early postmortem temperature with liquid nitrogen was shown to reduce protein denaturation and the formation of PSE pork (Borchert and Briskey, 1964). Furthermore, the WHC of pork was improved when pork carcasses were subjected to an accelerated air chilling system (Taylor and Dant, 1971; Kerth et al., 2001). Conversely, a number of studies have demonstrated that accelerated chilling at temperatures between −20°C and −40°C did not affect pork WHC (Gigiel and James, 1984; Gigiel et al., 1989; Jones et al., 1993; D'Souza et al., 1998a; Milligan et al., 1998).

The lack of an effect of accelerated chilling on WHC may be that increasing the chilling rate probably does not overcome the detrimental effects of preslaughter stress because the rapid pH decline associated with accelerated postmortem glycolysis may be complete well before entering the chilling cooler (Offer, 1991; Hambrecht et al., 2004a). Furthermore, if carcass temperature is reduced too rapidly, sarcomere shortening can occur when muscle ATP concentrations are still high (Locker and Hagyard, 1963). And, as mentioned previously, WHC decreases with decreasing sarcomere length (Honikel et al., 1986; Bertram et al., 2002).

Electrical Stimulation

Electrical stimulation of beef carcasses has been shown to increase tenderness by reducing cold-shortening (Davey et al., 1976), produce brighter colored lean with more visible marbling, and reduce the need for long-duration of postmortem aging periods (Savell et al., 1977, 1978). Electrical stimulation also improves pork tenderness (Bowker et al., 1999); however, electrical stimulation accelerates postmortem glycolysis and increases the incidence of PSE pork (Hallund and Bendall, 1965; Gigiel and James, 1984; Bowker et al., 1999; Maribo et al., 1999). Even though Martin et al. (1983) reported that electrical stimulation improves the WHC of beef, most research has either failed to note an effect of electrical stimulation on WHC (McKenna et al., 2003) or actually reported reductions in the WHC of beef in response to electrical stimulation (Unruh et al., 1986; den Hertog-Meischke et al., 1997).

Methods of Measuring the WHC of Meat

Water-holding capacity can be predicted by measuring drip loss, either by the gravitational method of Honikel et al. (1986) or the Danish drip tube method developed by Rasmussen and Andersson (1996). On the other hand, the proportion of bound water can be measured via the application of centrifugal force (Wierbicki et al., 1956, 1957a, 1957b; Bouton et al., 1971, 1972; Penny, 1975; Jauregui et al., 1981) or modifications of the filter-paper press method of Grau and Hamm (1953).

Gravitational Drip Loss

Honikel (1997, 1998) has proposed a standardized, gravitational method of measuring drip loss in raw, whole muscle that requires: (1) a balance capable of weighing to the nearest hundredth or thousandth (0.01–0.001) with an accuracy of ± 0.05 g; (2) sealable, water-impermeable (plastic) bags; and (3) sample support that allows the escape of fluid (plastic net bag, fishhook with barb removed, etc.). Basically, remove and weigh an 80–100 g sample of meat, and suspend it either in netting or on a barbless fishhook in an inflated bag (please ensure that the sample does not come into contact with the bag or container). After 24–48 hours of storage at 1–4°C, samples are removed from bags and suspension device (netting or hook), gently blotted dry on paper towels, and subsequently weighed. The difference between the initial and final sample weights is divided by the initial sample weight to calculate drip loss percentage.

There have been a number of modifications to this particular gravitational method. In general, 3.8- to 4.0-cm-thick steaks/chops are removed perpendicular to the muscle fiber orientation, and duplicate 3.8-cm-diameter cores should be removed from the center of each section (must ensure that each sample is cut in such a manner to maintain a constant volume-to-surface area). The core(s) should be weighed to the closest 0.01 g and suspended on an S-hook (recommend fishing hooks with barbs removed). Then, S-hooks can be hung within inflated plastic bags similar to the recommended, standardized method, or hung within large, airtight containers with holes drilled in the lid (allow plenty of space so that samples do not touch each other when hung within the container). Again, after a 48-hour storage period at 0–4°C, remove samples from S-hooks, blot dry on paper towels, and reweigh to calculate drip loss percentage.

Drip Tubes

This relatively new procedure (called "EZ-DripLoss") of Rasmussen and Andersson (1996) is routinely used by the Danish Meat Research Institute to measure drip loss in pork. Moreover, both Christensen (2003) and Otto et al. (2004) have shown that the EZ-DripLoss method is highly correlated with the gravitational method, has greater sensitivity, is much easier, and is less time consuming to perform.

The procedure requires that at least three, 25-mm-diameter cores be removed from 2.54-cm-thick steaks/chops and weighed. Then, the individual cores are placed into preweighed, funnel-shaped plastic "drip" tubes (Sarstedt Inc., Newton, NC; Christensen Aps Industrivaengetand, Hilleroed, Denmark; KABE Labortechnik, Nümbrecht-Elsenroth, Germany) before storage at 4°C for 48 hours. Similar to the "bag" gravitational method, cores are removed and reweighed, and differences in weight are used to calculate drip loss percentage. It should be noted that "dabbing" (or blotting) meat samples of visible moisture before weighing was found to increase the accuracy of the EZ-DripLoss method when compared to the Honikel "bag" suspension method for measuring pork drip loss (Correa et al., 2007).

Filter-Paper Press

At one period of time, the filter-paper press method was the standard for measuring the WHC of fresh, frozen, and cooked meats. The original method of Grau and Hamm (1953) was modified

slightly by Wierbicki and Deatherage (1958) and Urbin et al. (1962), and remains a relatively simple procedure that requires: (1) a desiccator containing saturated potassium chloride pellets; (2) 5.5- to 7.0-cm-diameter filter paper (preferable Whatman no. 1 or Whatman no. 50); (3) forceps to handle paper; (4) scale that can weigh to the nearest ten-thousandths (0.0001 g); (5) two 1.27-cm-thick, 12.7 × 12.7 cm sheets of Plexiglas or light-weight aluminum; (6) Carver press (Fred S. Carver, Inc., Summit, NJ) or other device that can repeatedly generate at least 35.2 kg/cm^2 (500 psi) of force; and (7) a planimeter. Lastly, the total moisture content of an additional sample of meat being tested must be measured either using the forced, oven-drying procedure (method no. 950.46; AOAC, 1995) or the freeze-drying procedure of Apple et al. (2001).

Take a single sheet of filter paper from the desiccator, and weigh it to the nearest 0.0001 g. Then, place 0.5 g of meat in the center of the filter paper and quickly reweigh filter paper and meat. Place the filter paper between the two sheets of Plexiglas (or metal sheets) and press for one minute at 35.2 kg/cm^2 (or 500 psi). Separate the Plexiglas sheets, carefully trace the inner (meat film layer; MFA) and outer circles (total surface area; TFA) formed on the filter paper, and measure the areas (in^2) of each circle with a compensating planimeter (recommend the Planix 8 from the Sokkia Corporation of Overland Park, KS).

First, the total moisture (in mg) is calculated by multiplying the sample weight (mg) by the total moisture content (%) of the sample. Then, the proportion of free (expressible) water (%) is calculated as: ((TFA – MFA) × 61.1) total moisture [mg]) × 100 (1 square inch is equivalent of 61.1 ± 0.31 mg of free/expressible moisture; Wierbicki and Deatherage, 1958); whereas, the proportion of bound water (%) is simply: 100 − free water (%).

Centrifugation

Several researchers have used centrifugation of intact and ground meat samples to measure the WHC of meat. Bouton et al. (1971, 1972) placed 3–4-g samples of whole muscle samples in stainless steel centrifugation tubes and spun the samples at 100,000 × *g* (36,000 rpm) for 1 hour, and calculated the percentage of bound water from the initial and post-centrifugation weights of the meat sample. However, other researchers have shown that using slower centrifugation speeds and centrifugation durations of 30 minutes or less, can elicit reproducible WHC measurements that are highly correlated ($r = 0.88$) with drip loss percentage (Penny, 1975).

Recent studies by Von Seggern et al. (2005) and Tschirhart-Hoelscher et al. (2006) have employed the centrifugal method of Jauregui et al. (1981) to calculate the expressible moisture of several muscles (Table 7.3). Basically, three pieces of 5.5-cm-diameter filter paper (Whatman no. 3) and a single piece of 7.0-cm-diameter filter paper (Whatman no. 50) are folded into a thimble shape over a 16 × 405 mm test tube (the Whatman no. 50 filter paper on the inside of the formed thimble). The filter paper thimble is weighed to the nearest 0.0001 g before the addition of 1.5 g of minced meat sample. The sample and thimble are then spun in 50 mL centrifuge tubes at 30,900 × *g* (16,000 rpm) for 15–30 minutes at 2°C. Remove the filter paper and sample from the centrifugation tubes, remove the meat "pellet" from the filter paper, and reweigh the pellet and filter paper. The difference between the initial and post-centrifugation meat sample weights can be divided by the initial sample weight, or the weight of the filter paper after centrifugation can be divided by the initial weight of the filter paper and sample, to calculate the percentage of expressible moisture.

Table 7.3 Expressible moisture content (%) of specific muscles

Muscle	Beef [a,b]	Pork [c,d]	Lamb [b,e]
Adductor	41.56	5.64	39.4
Biceps femoris	40.81	7.74	38.1
Complexus	36.37	–	–
Gluteus medius	37.60	7.74	37.7
Infraspinatus	38.48	3.80	32.4
Latissimus dorsi	37.26	4.50	29.4
Longissimus thoracis et lumborum	37.75	7.71	39.7
Pectoralis profundi	39.02	5.83	29.3
Psoas major	–	5.91	34.8
Rectus femoris	40.33	4.19	35.6
Semimembranosus	40.77	8.16	37.4
Semitendinosus	39.06	8.23	31.3
Serratus ventralis	36.55	5.74	31.7
Supraspinatus	39.67	5.13	33.4
Tensor fasciae latae	39.06	3.98	32.7
Teres major	38.64	–	34.8
Triceps brachii	40.21	4.52	29.2
Vastus intermedius	39.29	4.26	–
Vastus lateralis	40.33	–	36.0
Vastus medialis	41.39	4.26	–

[a] Von Seggern et al. (2005).
[b] Expressible moisture measured using the centrifugation method of Jauregui et al. (1981).
[c] Porcine Myology (www.porcine.unl.edu).
[d] Expressible moisture measured by centrifugation at 4,500 × *g* for 10 min (S.M. Lonergan, personal communication).
[e] Tschirhart-Hoelscher et al. (2006).

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8 Lipids and Lipid Oxidation

Kyle Willian

Introduction

In this chapter, the general structure and classification of the lipids will be presented with emphasis on those found in muscle tissue. In addition, degradation of lipids (i.e., oxidation) in muscle foods will be presented since this directly affects meat quality. The extraction and analysis of these lipid components will also be discussed. As not all types of lipids (e.g., various types of fatty acids) are commonly found in muscle, this chapter will be specific to the most commonly analyzed types of lipids. The biological function of lipids will not be presented in that the goal here is to understand the physical and chemical properties of lipids, and how those properties are important for analyses. The most common type of information required in lipid analysis of meat is to ascertain the total fat content, fatty acid profile, amount of lipid degradation (oxidation), cholesterol, certain fat soluble vitamins, and perhaps other lipid nutrients such as carotenoids. Therefore, these components will be the focus of this chapter. Several of the most common methods available for lipid analysis will be discussed and at the end of the chapter, recipes for some procedures are given. The few procedures provided are those used by the author. It is important to point out, however, that literally hundreds and hundreds of variations of the methods presented at the end of the chapter have been published. The goal of presenting just the few that we have used in our laboratory is that a first-time investigator will have a starting point. The reader is heavily encouraged to read the literature and investigate the many methods available.

Structure, Nomenclature, and Classification of Meat Lipids

Lipids can be broadly defined as organic compounds (mostly consisting of hydrogen, carbon, nitrogen, oxygen, and phosphorus) that are virtually insoluble in aqueous solutions but highly soluble in organic solvents, most commonly, dichloromethane, chloroform, hexane, and diethyl ether. Lipids are different from the other major biomolecules, carbohydrates, proteins, and nucleic acids, because these compounds are mostly soluble in aqueous media and insoluble in organic solvents. Lipids include such compounds as fatty acids, triacylglycerols, waxes, glycerophospholipids, sphingolipids, sterols, terpene-derived molecules such as carotenoids, and fat-soluble vitamins: A, D, E, K, and their derivatives. Lipids are also different from polysaccharides, proteins (polypeptides), and nucleic

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acids because they are not polymers of smaller monomer units. They are, however, composed of smaller building blocks such as glycerol, fatty acids, and phosphoric acid. Lipid components can also be covalently attached to proteins and mono- or oligosaccharides. Because of this mixture of polar and nonpolar components, many lipids display amphipathic behavior, that is, they have hydrophobic and hydrophilic components. Lipids can generally be classified as either neutral lipids (nonpolar) or those that are amphipathic.

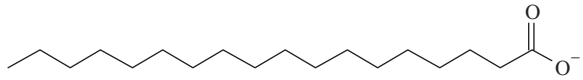
Fatty Acids

A fatty acid consists of a hydrocarbon chain (an alkyl chain) that has a single carboxyl group at one end. Fatty acids are either saturated, containing no double bonds, or unsaturated, having one or more double bonds. They are often split into the following categories: short-chain fatty acids (SCFA) with only 2–8 carbon atoms, medium-chain fatty acids (MCFA) with 10–16 carbons, and the long-chain fatty acids (LCFA) with more than 16 carbons. Fatty acids that have 20 or more carbon atoms are also frequently referred to as the very long chain fatty acids (VLFA). Fatty acids can further be referred to by their degree of unsaturation: monounsaturated (MUFA) or polyunsaturated fatty acid (PUFA). Thus, as an example, a fatty acid such as docsopentaenoic acid, 22:5n3, is a very long chain polyunsaturated fatty acid (VLCPUFA).

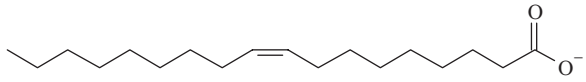
The basic structure of fatty acid is shown in Figure 8.1 that displays the 18-carbon series molecules. Below each molecule in Figure 8.1, the common name is listed, followed by the International Union of Pure and Applied Chemistry (IUPAC) name, and then other naming systems. In the IUPAC method for naming fatty acids, each carbon chain length has a designated name, for example, decanoic acid (or decanoate) for 10 carbons, hexadecanoic acid (hexadecanoate) for 16 carbons. Double bonds are designated by the delta system (Δ), identifying their position starting by counting carbon atoms from the carboxyl end of the molecule (refer Fig. 8.1). Each double bond position is further designated as to whether it is in a *cis* or *trans* conformation. Since the carbon double bonds in most fatty acids are *cis*, the *cis* designation is usually not included and only when there is a *trans* bond it is called out. Fatty acids have several other nomenclature systems and common names. The most common naming system is the omega (ω) or n-system. In this system, the ω symbol or the *n* represents the same thing which is to designate the first carbon counting from the methyl end of the molecule where a double bond occurs. The number after the ω or *n* tells how many double bonds are present. The *dashed arrows* in Figure 8.1 are shown because mammals lack the enzymes necessary to insert double bonds at the n6 position or before. Only plants have the enzymes required to make ω6 and ω3 fatty acids, and since mammals require these lipids, linoleic and α-linolenic are the essential fatty acids in the mammalian diet.

Although typically present in only trace amounts, there are two other kinds of fatty acids found in meats: conjugated and branched-chain fatty acids (BCFA). Most naturally occurring fatty acids are not conjugated, that is, in between each set of double bonds is an sp^3 hybridized methylene carbon atom. The short-hand notation (*n*, ω) cannot be used to name fatty acids that contain conjugated double bonds. An example of a conjugated fatty acid is *cis,trans*-9,11 octadecadienoic acid, as shown at the bottom of Figure 8.1. *Cis,trans*-9,11 octadecadienoic acid is an example of a conjugated linoleic acid (CLA) molecule.

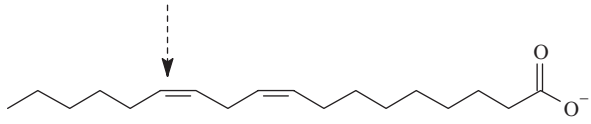
BCFA have two basic isomers: the *iso* and the *anteiso* forms, where a methyl group is found at the second to last carbon of the chain or the third to the last carbon. Figure 8.2 shows examples of BCFA with 15 carbons in its *iso* form, 14-methyl tetradecanoic acid, and the *anteiso* isomer, 13-methyl tetradecanoic acid.



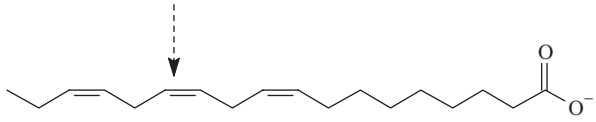
Stearic acid, octadecanoic acid, 18:0



Oleic acid, 9-octadecenoic acid, 18:1ω9, 18:n9, 18:1Δ9

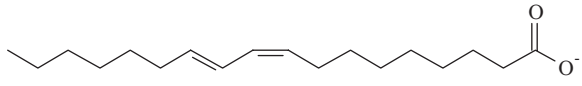


Linoleic acid, 9, 12-octadecadienoic acid, 18:2ω6, 18:n6, 18:2Δ9, 12



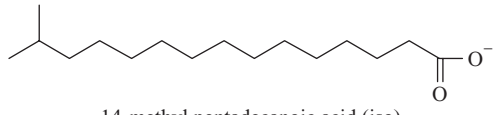
α-Linolenic acid, 9, 12, 15-octadecatrienoic acid, 18:3ω3, 18:n3, 18:3Δ9, 12, 15

ω or n ⟶ ⟵ Δ

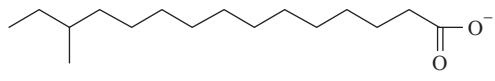


cis,trans 9, 11-octadecadienoic acid, 18:2Δc9, t11

Figure 8.1 The 18-carbon series of fatty acids. Common, systematic, and short-hand nomenclature is provided. The molecule at the bottom is a less common fatty acid, a type of conjugated linoleic acid (CLA) molecule that is shown with one trans-double bond.



14-methyl pentadecanoic acid (iso)



13-methyl pentadecanoic acid (anteiso)

Figure 8.2 Examples of branched-chain fatty acids (BCFA). Both fatty acids pictured are pentadecanoates but one is the iso form and the other is the anteiso form.

Table 8.1 Fatty acids commonly found in meats and their relative abundance

Short-hand notation	Common name	IUPAC	Weight percent
14:0	Myristic	Tetradecanoate	1–2%
14:1n5, Δ^{9}		9-tetradecenoate	<1%
15:0		Pentadecanoate	<1%
15:1n5, Δ^{9}		9-pentadecenoate	<1%
16:0	Palmitic	Hexadecanoate	20–25%
16:1n5, Δ^{9}	Palmitoleic	9-hexadecaenoate	1–3%
17:0		Heptadecanoate	<1%
17:1n, Δ^{9}		Heptadecenoate	<1%
18:0	Stearic	Octadecanoate	10–15%
18:1n9, Δ^{9}	Oleic	9-octadecaenoate	25–35%
18:1n7, Δ^{11}	Vaccenic	11-octadecaenoate	1–2%
18:1n9t, Δ^{9}	Eladiac	Trans-9-octadecaenoate	1–2%
18:2n6, $\Delta^{9,12}$	Linoleic	9,12-octadecadienoate	2–10%
18:3n3, $\Delta^{9,12,15}$	α-Linolenic	9,12,15-octadecatrienoate	<1%
18:3n6, $\Delta^{6,9,12}$	γ-Linolenic	6,9,12-octadecatrienoate	<1%
19:0		Nondecanoate	<1%
19:1		Nondecenoate	<1%
20:0	Arachidic	Icosanoate	<1%
20:1n9, Δ^{11}	Gondoic	11-icosenoate	<1%
20:1n11, Δ^{9}	Gadoleic	9-icosenoate	<1%
20:3n6, $\Delta^{8,11,14}$	Dihomo-γ-linolenic	8,11,14-icosatrienaote	<1%
20:2n6			<1%
20:3n9, $\Delta^{5,8,11}$	Mead	5,8,11-icosatrienaote	<1%
20:4n6, $\Delta^{5,8,11,14,}$	Arachidonic	5,8,11,14-icosatetraenaote	<1%
20:5n3, $\Delta^{5,8,11,14,17}$	Timnodonic (a.k.a. eicosapentaenoic, EPA)	5,8,11,14,17-icosapetaenaote	<1%
22:0		Docosanoate	<1%
22:1		Docosenoate	<1%
22:2		Docosadienoate	<1%
22:4n6, $\Delta^{7,10,13,16}$	Adrenic	7,10,13,16-docosatetraenoate	<1%
22:5n3, $\Delta^{7,10,13,16,19}$	Docosapentaenoic	7,10,13,16,19-docosapentaenoate	<1%
22:5n6, $\Delta^{4,7,10,13,16}$	Docosapentaenoic	4,7,10,13,16-docosapentaenoate	<1%
22:6n3, $\Delta^{4,7,10,13,16,19}$	Clupanodonic (a.k.a. docosahexaenoic, DHA)	4,7,10,13,16,19-docosahexaenoate	<1%
24:0	Lignoceric	Tetracosanoate	<1%
24:1	Nervonic	15-tetracosenoate	<1%

Table 8.1 lists the major fatty acids over the range of 14–24 carbon atoms. The table provides short-hand notations, common names (if applicable), systematic names, and the approximate weight percent of each fatty acid found in the meat from typical land-based animals (beef, poultry, pork, etc.). Although aquatic animals have many of the same fatty acids as land-based animals, they typically have quite different fatty acid profiles especially in seafood. Table 8.1 highlights some very important aspects of fatty acid nomenclature. For example, two fatty acids of major nutritional importance are 4,7,10,13,16,19-docosahexaenoate and 5,8,11,14,17-icosapetaenoate. These two fatty acids are rarely discussed in the literature by their common or systematic names and are almost always referred to as simply docasahexaenoic acid (DHA, 22:6n3) and eicosapentaenoic acid (EPA, 20:5n3). In contrast, the shorter 14–18 carbon fatty acids are typically called by their common names: oleic, linoleic, palmitc, etc. Also notice that both 4,7,10,13,16-docosapentaenoate (22:5n3) and 7,10,13,16,19-docosapentaenoate (22:5n6) share the common name docosapentaenoate. It is

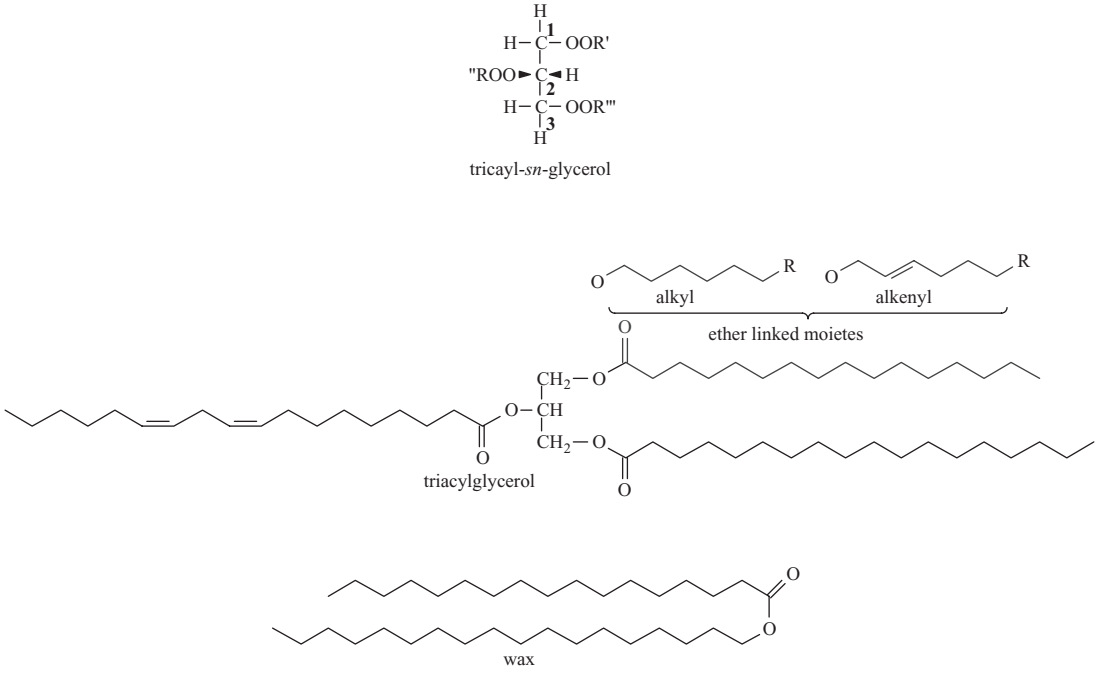


Figure 8.3 The neutral lipids. Triacylglycerols, ether-linked glycerols called alkyl or alkenyl ethers, and waxes. The molecule at the top shows glycerol in a Fisher projection with the *sn*-number system.

very important, therefore, to become familiar with all of the various naming systems and to be careful when reading the scientific literature about fatty acids.

Neutral Lipids

The neutral lipids consist of the triacylglycerols, diacylglycerols with ether-linked groups called alkyl and alkenyl ethers, and waxes as shown in Figure 8.3. In waxes, the carboxyl group on fatty acids is reduced to a hydroxyl and then esterified to a fatty acid to form a wax. Although waxes are found in both plants and animals, they are not, however, found in muscle tissue.

Triacylglycerols are made from a glycerol molecule where each hydroxyl group has been replaced to form an ester linkage with the carboxyl group of a fatty acid. They are the main storage form of fatty acids in adipose tissue. The positions on the glycerol moiety are designated as the *sn*1, *sn*2, or *sn*3 positions based on the Fisher projection of glycerol as shown in Figure 8.3. A triacylglycerol composed of palmitc, linoleic, and stearic acids is also displayed in Figure 8.3. This compound would be named 1-palmitoyl-2-linoleicoyl-3-stearoyl-*sn*-glycerol. Unsaturated fatty acids, when present, are found on *sn*2 position. Triacylglycerols are mainly stored in specialized cells, adipocytes. In other cells, such as the myocytes, small amounts are found in the form of droplets that are dispersed in the cytosol.

In alkyl and alkenyl ethers, a long hydrocarbon unit is attached to a glycerol molecule via an ether linkage. These ether-linked species are similar to triacylglycerols, but the fatty acid at the *sn*1 position on the glycerol molecule is replaced by the alkyl or alkenyl molecule making a

diacylglycerol with the ether-linked lipid. Figure 8.3 also shows the structure of a triacylglycerol and what it would look like with an alkyl or alkenyl group attached. These ether-linked glycerolipids are called 1-alkyl-2,3-diacylglycerol or 1-alkenyl-2,3-diacylglycerol. If the specific fatty acids at the *sn*2 and *sn*3 positions need to be identified that would be done similar to the naming in a triacylglycerol.

Polar Lipids

Glycerophospholipids are the main components of the membrane of the cell and cellular organelles and thus they contribute greatly to the total lipid extract in muscle tissue. As shown in Figure 8.4, the glycerophospholipids are composed of a glycerol molecule esterified with two fatty acids and a phosphodiester linkage to a polar head group such as ethanolamine. The simplest is phosphatidic acid found in only very small amounts in cellular membranes. Most cellular membranes consist of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phsophatidylinositol (PI), and phosphatidylserine (PS), followed by diphosphotidylglycerols commonly referred to as cardiolipids. Diphosphotidylglycerols were first discovered in cardiac tissue and thus the name cardiolipids; however, they are present in small amounts in all membranes such as muscle. If the polar head group in the molecule shown in Figure 8.4 was a choline, then this glycerophospholipid would be named 1-palmitoyl-2-linoleoyl-*sn*-glycerol-3-phosphocholine.

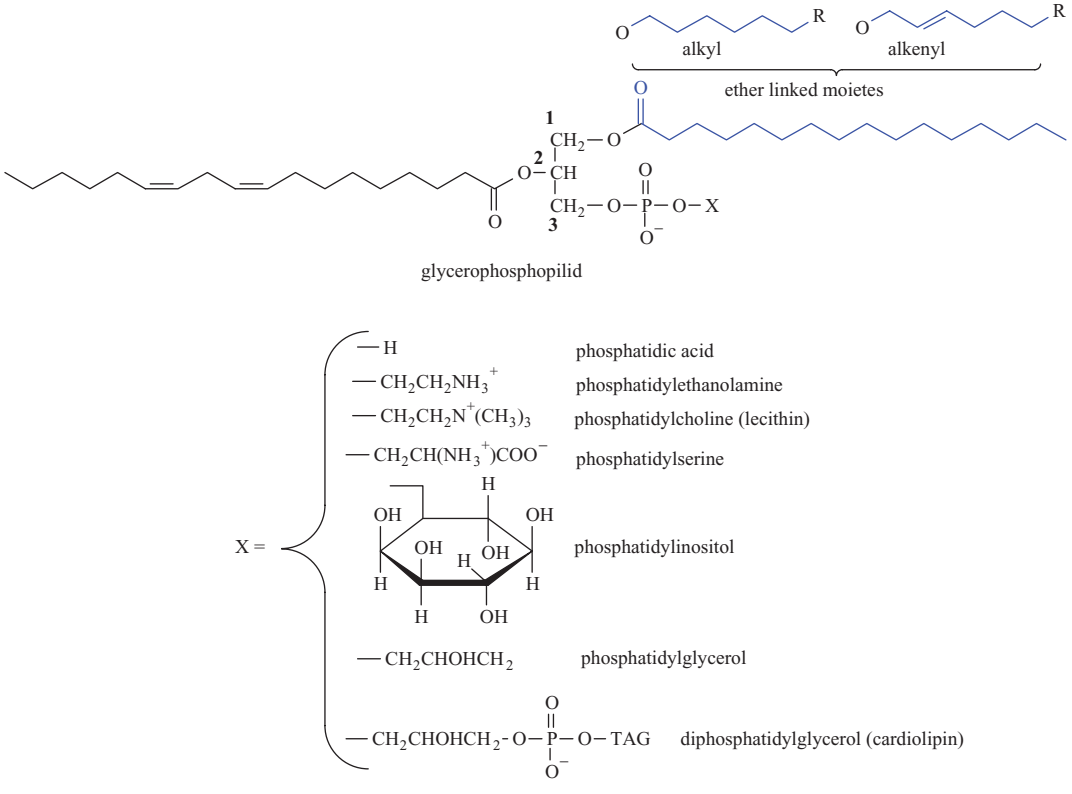


Figure 8.4 The glycerophospholipids. The abbreviation TAG is used to represent another triacylglycerol in the structure of the cardiolipin. The alkenyl ether analogs of phosphotidylcholine are called plasmologens.

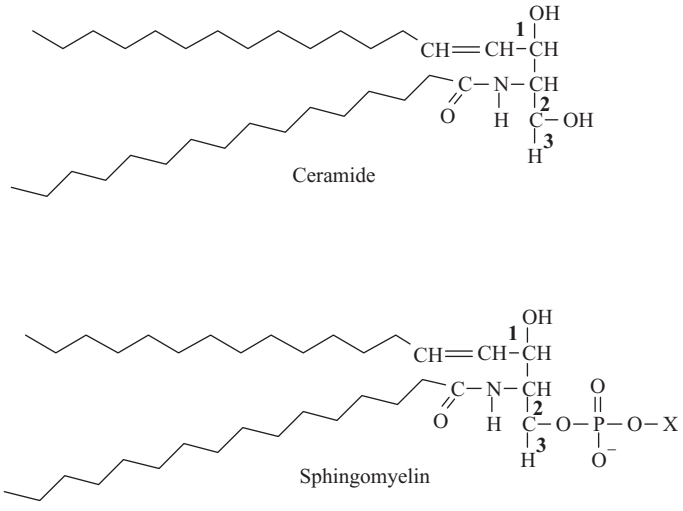


Figure 8.5 The ceramides and sphingolipids. It is important to note that the fatty acid attached at the *sn*2 position is *N*-acyl linked.

Plasmalogens have the basic glycerophospholipid structure but an α,β-unsaturated ether linkage is found at the *sn*1 position on the glycerol while in ether phosphatides there is an alkoxy ether connection.

The sphingolipids consist of a ceramide core structure with a fatty acid moiety attached via an *N*-acyl linkage as shown in Figure 8.5. The most common ceramides are the sphingomyelins where either a phosphoethanolamine or phosphocholine group is attached. Other ceramides are called cerebrosides and gangliosides.

The isoprenoids (also called terpenoids) are organic soluble molecules that are derived from the basic building block, isoprene, as shown in Figure 8.6. Thousands of isoprenoids have been found in nature from virtually all living organisms. The fat-soluble vitamins, A, D, E, and K, cholesterol and other sterols, and plant phytochemicals such as the carotenoids (e.g., β-carotene, lycopene) are all biochemically synthesized from the isoprene unit. The discussion from this point will be limited to cholesterol and vitamins A and E because quantification of these nutrients has direct impact on human health and nutrition. Vitamins D and K are important to human nutrition but current research is focused on supplementing production animal diets with vitamins A and E and further vitamins D and K can also be made endogenously in humans. In addition, vitamin E, and to a somewhat lesser extent, vitamin A are important antioxidants found in the meat that prevent oxidation and rancidity.

Vitamin A refers to a collection of molecules, retinal, retinol, or retinoic acid, as seen in Figure 8.6. In animal foods, it is found as a retinyl ester, primarily retinyl-palmitate, which is hydrolyzed in the small intestine to retinol. Vitamin A is commonly measured and reported in international units (IU) of retinol, and 1 IU = 0.3 μg retinol. Retinol is found in most muscle foods at around 0.1–0.2 μg/g, although in certain fish species it can be as high as 1.0–2.0 μg/g (USDA, 2008).

Vitamin E refers to a group of eight related molecules, four tocotrienols, and four tocopherols, as shown in Figure 8.6. The most abundant forms of vitamin E are the tocopherols with α-tocopherol the predominant and most biologically active. The position marked with the *star*, H, in Figure 8.6 shows an anomeric position and so there are two isomers for each molecule, D and L. The D form is

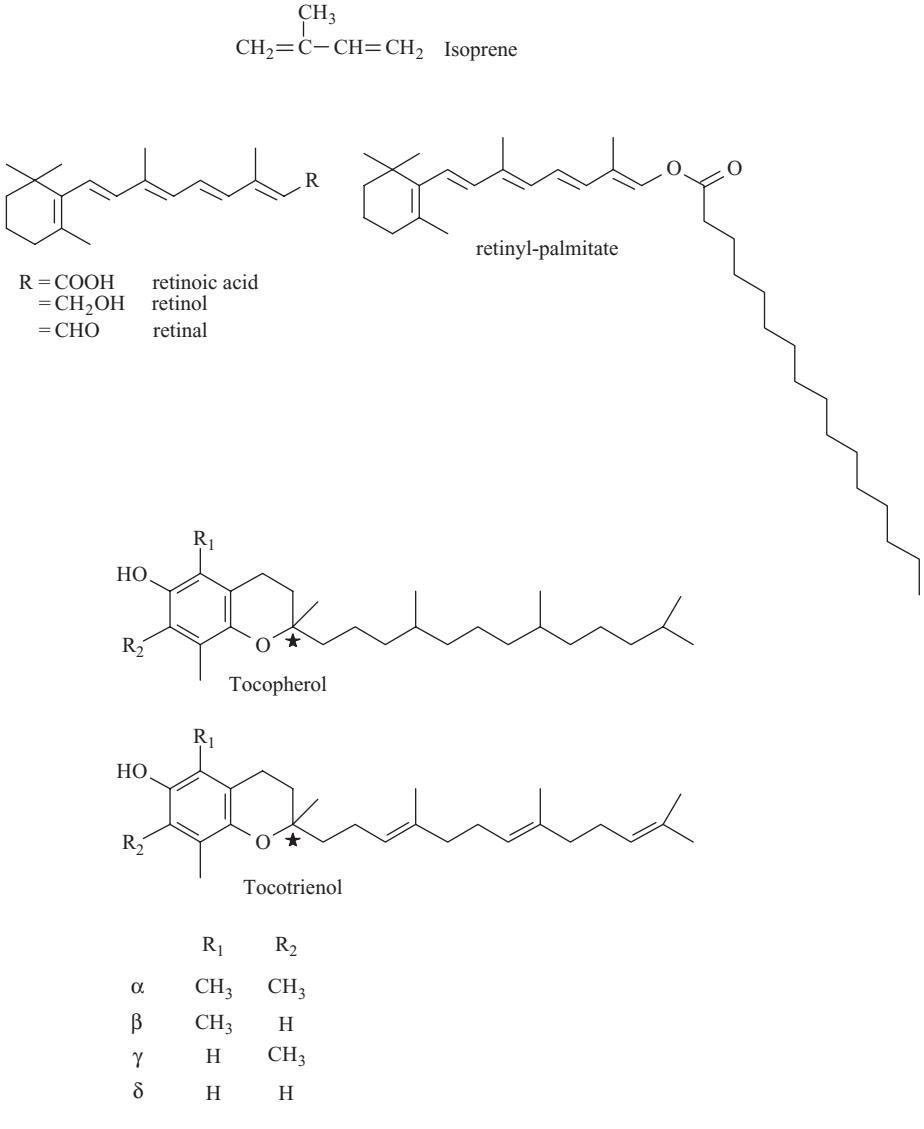


Figure 8.6 The fat-soluble isoprenoid vitamins A and E.

the more biologically active species and as such vitamin E is commonly reported in IU where 1 IU of vitamin E = 0.667 mg D-α-tocopherol. With respect to D-α-tocopherol, L-α-tocopherol has only 26% as much biological activity, D-α-tocotrienol has 22%, and DL racemic mixtures of tochoperol and tocopherol acetates have around 70% as much activity. The amount of D-α-tocopherol in meat is typically 1–5 μg/g, but once again in some fish species there can be 4 or 5 times this amount (USDA).

Under this category are also sterols, since they are composed of isoprene units as well, the fundamental example being cholesterol. Cholesterol is synthesized virtually in all vertebrate animals but not in plants. Plants make their own form of sterols called phytosterols. Cholesterol can be

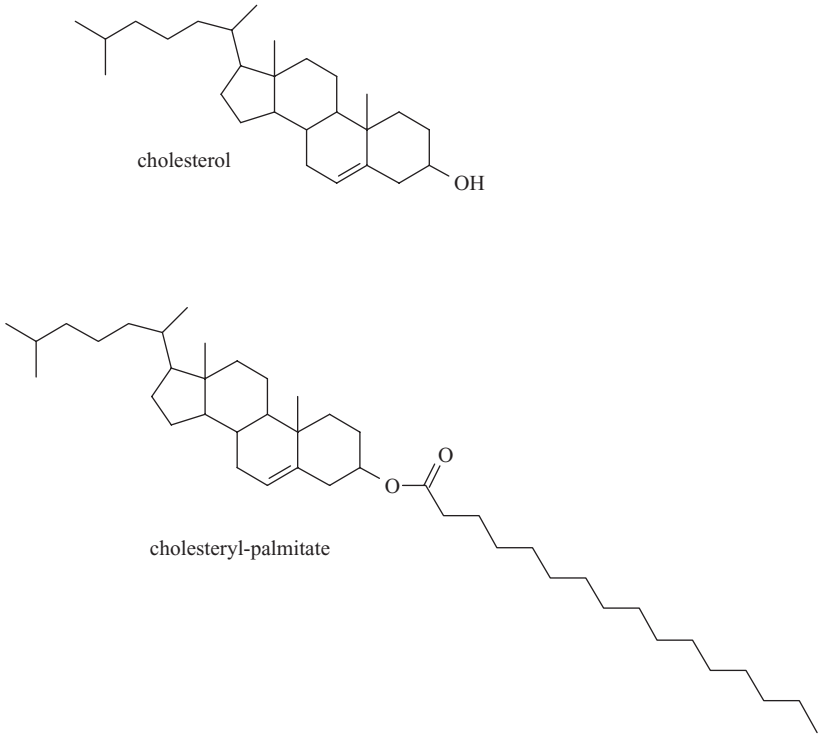


Figure 8.7 Cholesterol and an esterified cholesterol.

a significant portion of the plasma phospholipid membrane of the cells (10–40%) and is a minor component in the membrane of the cellular organelles. In membranes it is typically found unesterified but in plasma lipoproteins it is 70% esterified to fatty acids, usually to palmitic acid as cholesteryl-palmitate, as seen in Figure 8.7. The amount of cholesterol in most meats is usually in the range of 50–100 mg/100 g (USDA).

Saponifiable and Nonsaponifiable Lipids

Lipids can also be broadly organized further into two categories based on their reaction to base hydrolysis: saponifiable and nonsaponifiable. Strictly speaking, the term saponification is hydrolysis of lipid molecules held by an ester linkage using a strong base such as NaOH or KOH resulting in the formation of the free fatty acid ionic salts as shown in Figure 8.8. Saponification cleaves off *O*-acyl linked fatty acids from triacylglycerols, glycerophospholipids, and sterol and vitamin esters. The glycerol-linked lipids, triacylglycerols and glycerophospholipids, are generally considered saponifiable, while sterols and terpene-derived molecules are considered nonsaponifiable. Obviously, this is not a strict definition since some sterols and vitamins are esterified but at the end of a saponification procedure all aqueous components such as the glycerol, phosphoglycerol, and fatty acids have been removed leaving only the very nonpolar lipids, fat-soluble vitamins, sterols, and other terpenes behind.

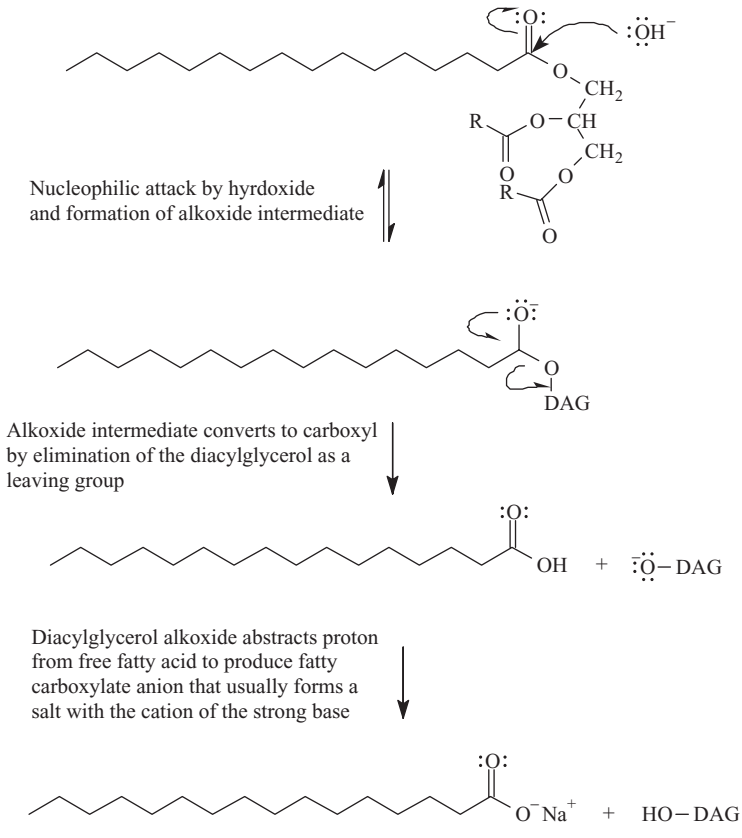


Figure 8.8 Saponification: hydrolysis of a triacylglycerol using a strong base such as NaOH. The abbreviation DAG is used here for diacylglycerol.

Composition of Lipids in Meat

In a typical meat sample, the amount of total lipids is usually in the range of 50–100 mg/g, although in fish there can be much greater variation 10–200 mg/g depending on species. On a smaller-scale analysis (using 1 or 2 g sample of muscle) as is usually done when assessing fatty acids, the amount of total lipids recovered is usually less, 10–60 mg of total lipid per gram of wet sample. The larger ranges of total lipids are based on analysis of whole cuts of different meats (USDA, 2008), while in a small analysis, a 1 g sample is usually used after it has carefully been excised of any visible fat. In an analysis of catfish, salmon, pork, beef, chicken, and turkey, the order of least to most total lipids was catfish $<$ turkey $<$ chicken $<$ salmon $=$ beef $<$ pork (Blank et al., 1992). Total phospholipids ranged from approximately 3.5 mg/g (pork) to 6.0 mg/g (chicken) or on a percent of total lipids, approximately 9% (pork) to 25% (chicken). The amount of phospholipids in the muscle stays relatively unchanged as the animal ages but the amount of triacylglycerol can increase dramatically. In steers, from 14 months of age to 24 months, the relative amount of phospholipids decreased from about 30% to about 10% of total lipids while in pigs starting from an initial weight of 40 kg the relative amount of phospholipids dropped from about 45% to about 30% over a 80-day feeding period (Wood et al., 2008). Thus, depending on the type of meat, age of the animal, and

other potential factors such as diet there are large variations in total lipids and phospholipids. On average, therefore, an investigator could expect anywhere from 10% to 50% of total lipids recovered in meats to consist of phospholipids. Among the various phospholipids there is also a good bit of variation (Blank et al., 1992; Erickson, 2008) but typically meats consist mostly of PC, PE, and PS with roughly 40%, 15%, and 10% of total phospholipids, respectively. Sphingomyelin makes up another ~5% of the phospholipids and the remaining phospholipids are of mixed ether-linked species, alyl and alkenyl ethers such as plasmologens and ether phosphatides, and diradyl-inositols and serines to name a few.

Extraction of Lipids

Total Lipids

Extraction of lipids from animal tissue usually begins by drying (freeze-dried typically) the sample or some other pretreatment such as digestion or homogenization of the sample in the presence of an organic solvent and ending with collection and removal of the organic layer. Digestion using hydrolysis with strong acid or base is often a useful pretreatment step. Hydrolysis is necessary as it cleaves lipids that are covalently linked (acylated) to protein and carbohydrate moieties and helps to separate emulsified fats.

The most common method for the total extraction of lipids from animal tissue is the use of a 2:1 chloroform:methanol (CM) extraction solvent, otherwise known as the Folch method (Folch et al., 1957) or the closely related Bligh and Dyer method (Bligh and Dyer, 1959). Soxhelt extraction is also used and it is the AOAC-recommended method; however, it is typically the most time-consuming. Each method has its pros and cons but for extraction from muscle foods, the Folch method has prevailed. Some investigators (Iverson et al., 2001; Pérez-Palacios et al., 2008) have shown that the Folch procedure produces the best results in muscle foods, and since it is the most commonly used method, it is the focus of this discussion.

Before attempting a Folch total lipid extraction it is highly recommended, just as Christie (1992) points out, that the original Folch paper should be read. It gives very specific directions and clearly explains the limitations to the procedure. The tissue should be homogenized in 20 × its volume of CM. Typically, a 1 g sample is homogenized in 20 mL of CM. After homogenization, the solution is filtered. This is followed by washing the CM homogenate with 0.2 × its volume with a salt solution (e.g., 0.1 M NaCl). This washing step is critical as this is where the nonlipid material will be removed. It is absolutely important that the overall ratio of chloroform:methanol:water is 8:4:3 during the wash. So, in the example above, the homogenate would consist of 13.3 mL chloroform, 6.7 mL methanol, and approximately 1 mL of water (i.e., assumed tissue is mostly water and therefore 1 g of tissue releases approximately 1 mL of water). Therefore, addition of 4 mL of salt solution would bring the water volume to total 5 mL, and the 8:4:3 ratio is maintained. The homogenate is mixed and allowed to separate into two phases, a lower phase that is approximately 60% of the total solution and an upper phase the remaining 40% of the solution. The composition of these phases is 3:48:47 chloroform:methanol:water in the upper phase while the lower phase is 86:14:1. The upper phase is carefully removed (by disposable pipette) and then sides of the tube that the homogenate is in and the top of the remaining lower phase is rinsed with a couple of milliliters of the pure upper-phase solvent. This rinsing procedure helps to ensure that any remaining nonlipid material is removed and the rinsing is repeated a couple of times.

In the Folch paper, an explanation is also provided on the effects that different salts and wash solutions and concentrations (e.g., NaCl, $MgCl_2$, $CaCl_2$) have on complete recovery of all lipids as well as removal of nonlipid components. In muscle tissue, approximately 2% of the total lipids will be lost in the aqueous wash. Some acidic phospholipids, however, are lost more readily in the wash and as much as 35% of species such as PS may not be recovered.

Nonsaponifiable Lipids

Extraction of the nonsaponifiable lipids requires only digestion under alkaline conditions and complete extraction of all lipids is not necessary. Digestion aids in extraction of lipids as it emulsifies fats and releases lipid moieties that are covalently linked to proteins and carbohydrates and helps, in general, to break up animal tissue. The digestion solution is then mixed with an organic solvent (hexane, diethyl ether) creating a biphasic extract and the organic phase is then collected. Long-chain alcohols, hydrocarbons, sterols, and lipid-soluble vitamins and compounds such as carotenoids that were free or esterified from the original sample will be found in the nonsaponifiable layer. Cholesteryl esters and *N*-linked acyl moieties require longer times and harsher conditions to hydrolyze, so the degree to which they are cleaved can vary (Christie, 1992).

Analyses

Fatty Acid Methyl Esters

The most common method for the analysis of the fatty acids is to convert the fatty acids to fatty acid methyl esters (FAMEs) and then separate them using gas chromatography (GC). Results can be reported as a percent of total fatty acids (weight percent) and/or by quantifying the specific amount of each fatty acid on a per mass of meat tissue, usually in mg/100 g of sample.

The methylation or transesterification process is also often poorly understood and again should be studied carefully before being attempted and for an in-depth analysis, the reader is again referred to Christie's work (Christie, 1992). During methylation, the fatty acid moieties are cleaved from triaclyglycerols, phospholipids, or any other lipid molecules to which they might be acetylated by a hydrolysis reaction. The free fatty acids are then acetylated to a methylene group creating the FAMEs. The process is carried out using either acidic (HCl, H_2SO_4, BF_3) or basic (KOH, NaOH, $NaOCH_3$) methanol solutions. Figure 8.9 shows the chemical reaction and mechanism for acid-catalyzed transesterification.

Investigators have addressed the pros and cons of the many methods available for methylation of the fatty acids and the effectiveness of base versus acidic transesterification (Christie, 1992; Rule, 1997; Murrieta et al., 2003). Acid transesterification will rapidly methylate free fatty acids and *O*-acyl linked fatty acids. *N*-acyl linked moieties are slow to methylate under acidic conditions requiring higher temperatures and times for complete reaction. However, if reaction conditions are not entirely free of oxygen, then the chances for artifact formation become greater. Acidic transesterification will also produce dimethylacetyls from plasmologens or other alphatic aldehydes. Acid transesterification is not recommended for full recovery of CLAs, however, as it causes significant loss and isomerization changes. Use of KOH is better than BF_3 or $NaOCH_3$ especially for CLAs, and acidic conditions will cause up to 20% loss of CLAs (Murrieta et al., 2003).

Base catalyzed transesterification will also rapidly produce FAMEs from *O*-acyl linked lipids but is not effective for free fatty acids. Further, *N*-acyl fatty acids and ether-linked species (e.g., alk-1-enyl, sphingomyelins) are also not transesterified under basic condition.

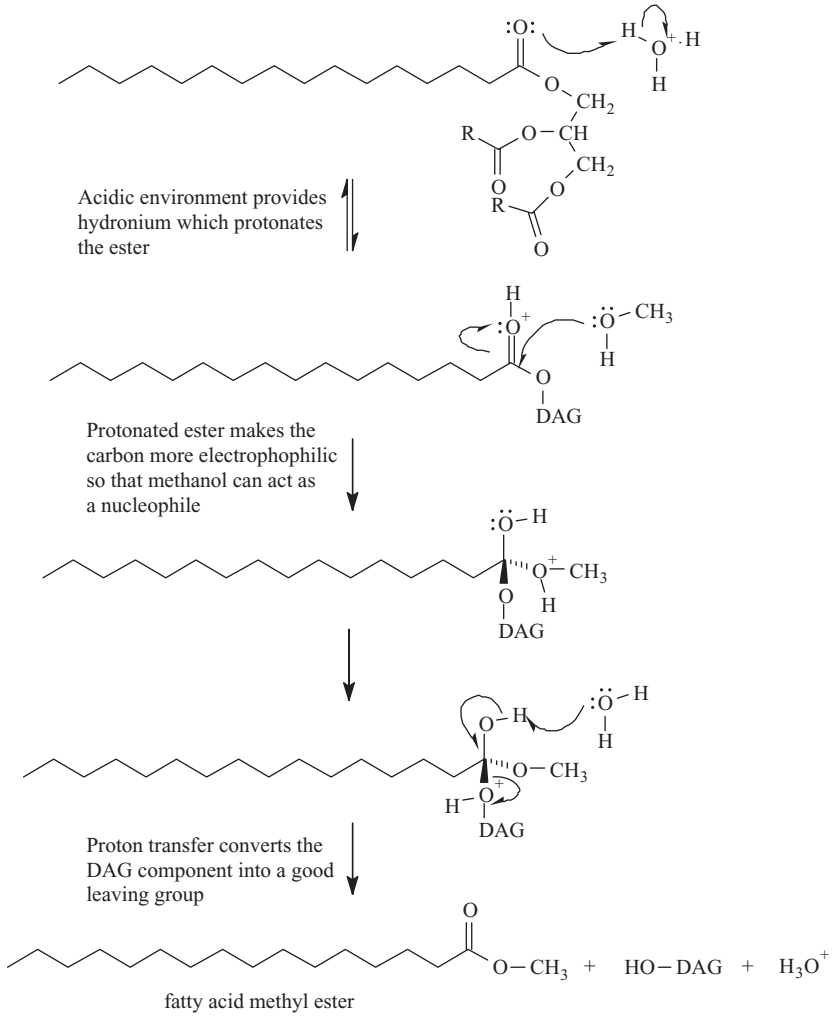


Figure 8.9 Acid catalyzed transesterification.

In both acid and base transesterification, the nonpolar lipids, triacylglycerols and especially cholesteroyl esters, are slow to produce FAMEs. In addition, these lipids are not very soluble in methanol and additional solvent is necessary to first solublize the nonpolar lipids by addition of a small amount (1 mL) of organic solvent such as toluene. If extensive agitation of the reacting solution, higher temperatures, and/or longer reaction times are employed, then the nonpolar lipids can interact with the reagents and most fatty acids are transesterified. Once again, however, longer reaction times and higher temperatures increase the chance for unwanted side reactions producing artifacts. For the small amount of lipid usually found in a small 1 g sample of muscle tissue, addition of extra organic solvent to solublize the nonpolar lipids is usually not necessary.

Methylation of the fatty acids can be done on the total lipids recovered after a Folch extraction, or direct transesterification from the meat matrix is also possible. If the initial total extraction of lipids can be avoided, much time is saved in the analysis. Direct transesterification of fatty acids

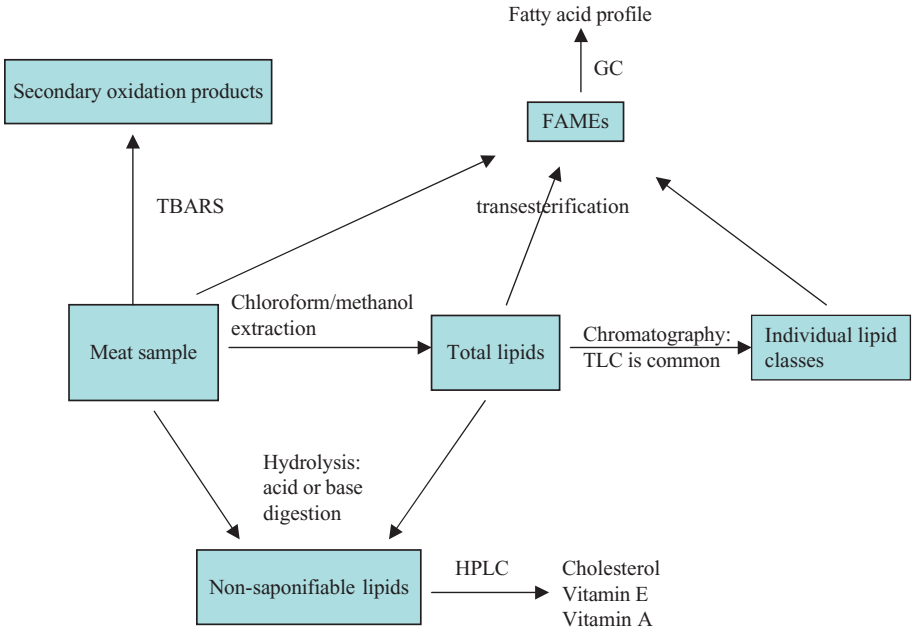


Figure 8.10 A block diagram of the steps in a typical analysis of meat.

from meats and other food products has been studied (Rule, 1997; Meier et al., 2006), and a recent investigation has shown that satisfactory results can be obtained from meat samples by a procedure using a base catalyzed reaction (KOH) followed by an acidic treatment (H_2SO_4) (O'Fallon et al., 2007), and this is the method of choice in the author's laboratory. If a true accounting of CLAs is required, however, only base catalyzed transesterification should be used and some investigators will subject the sample to both a basic and an acidic methylation procedure separately.

Figure 8.10 shows a block diagram of the procedures discussed to this point and the steps available in an analysis of a meat sample. As shown, the isolation of the nonsaponifiable lipids or FAMEs can be done directly to the meat sample. Unless the investigator needs to separate the lipids into all of the various classes, direct digestion, or transesterification of the meat sample is the simplest procedure. Collection of total lipids, however, allows the investigator to perform further chromatography, most often thin layer chromatography (TLC), in which all of the different lipid types can be separated. Free fatty acids, sphingomyelin, each of the phospholipids types, PE, PC, PI, PS, lysophosphotidyl choline, cholesterol, cholesteryl esters, tri-, di-, and monoacylglycerols, to name a few, can all be separated using TLC or simple silica gel column procedures. These methods will not be discussed here but the reader is encouraged to see the wonderful text by Christie (1992).

Chromatography

In this section, it will be assumed that the reader has some basic knowledge of chromatography and of the specific methods of GC and high-performance liquid chromatography (HPLC). A good source to review the basic principles would be in a quantitative analysis or instrumental analysis textbook (see e.g., Skoog, 2007). Some important terms to review, however, are the mobile phase,

stationary phase, and resolution. The mobile phase is the solvent (or gas in the case of GC) which carries the analyte of interest through the stationary phase. The stationary phase is the matrix upon which the analyte interacts. The degree to which the analyte interacts with the stationary phase determines the time it takes for the analyte to pass through the chromatography medium. If the analyte stays in the mobile phase more than it is attracted to and interacts with the stationary phase, then it elutes quickly. An analyte that spends more time on the stationary phase, therefore, elutes more slowly. The ability of a column to separate two different analytes is measured by the resolution (R_s). A resolution of 1.0 will allow for adequate quantification but for complete baseline resolution (no overlap of peaks) $R_s = 1.5$. An example of the calculation of resolution between two analytes will be presented in the analysis of a GC chromatogram of beef FAMEs below.

Another important concept, applicable to all forms of chromatography, is to understand the use of standards, both internal and external, for quantitative analysis. An internal standard is a compound that mimics the behavior of the analyte of interest during the chromatographic separation. The internal standard should have similar physical and chemical characteristics to the analyte and elute at close to the same time as the analyte. The standard needs to elute, however, just differently enough from the analyte of interest with $R_s > 1.25$. For an internal standard, a very carefully measured amount is added to the sample and then the peak area ratios of the standard and analyte are used. Use of an internal standard is recommended because it accounts for the uncertainty associated with the volume injected into the chromatography system from sample to sample. Another good reason to use an internal standard is that it can account for the loss of analyte during preparation (extraction, transesterification, etc.) of the sample. If the internal standard is added at the very beginning of an analytical procedure, then it can be assumed that loss of analyte and internal standard is similar. An example of using an internal standard will be presented below in the analysis of a GC chromatogram of beef FAMEs.

An external standard is a carefully prepared amount of the actual analyte and it is not added to the sample. The peak ratios of the analyte and from the external standard are, however, used to determine the amount of analyte in the sample just as with an internal standard. A series of external standards can also be made over a range of concentrations similar to the amount of analyte in the sample and plot of peak area as function of concentration can be used to make a calibration curve. A calibration curve is preferred as opposed to using a single external standard measurement. In the initial phases of method development, use of both an internal and external standard is recommended. If similar quantitative results (coefficient of variation $\leq 5\%$) are obtained, then one or the other standard can be used.

Gas Chromatography

Current gas chromatography (GC) of FAMEs is done almost exclusively using fused-silica capillary columns with a flame ionization detector (FID) and thus this combination will be the focus of this section. It should be stated, however, that GC coupled to a mass spectrometer is common, but for routine analysis of FAMEs most work is done with an FID. An FID is also a significantly cheaper detector to purchase and maintain. The gases used for the mobile phase in GC are nitrogen, helium, or hydrogen. Although more expensive, helium is usually the choice as nitrogen does not perform as well as helium. Hydrogen and helium have similar performance characteristics, but many investigators are concerned about the flammability of hydrogen. Hydrogen is, however, becoming more common as the price of helium continues to rise and many GC-column and GC-instrument manufacturers use hydrogen as the carrier gas. Since the performance of hydrogen and helium are

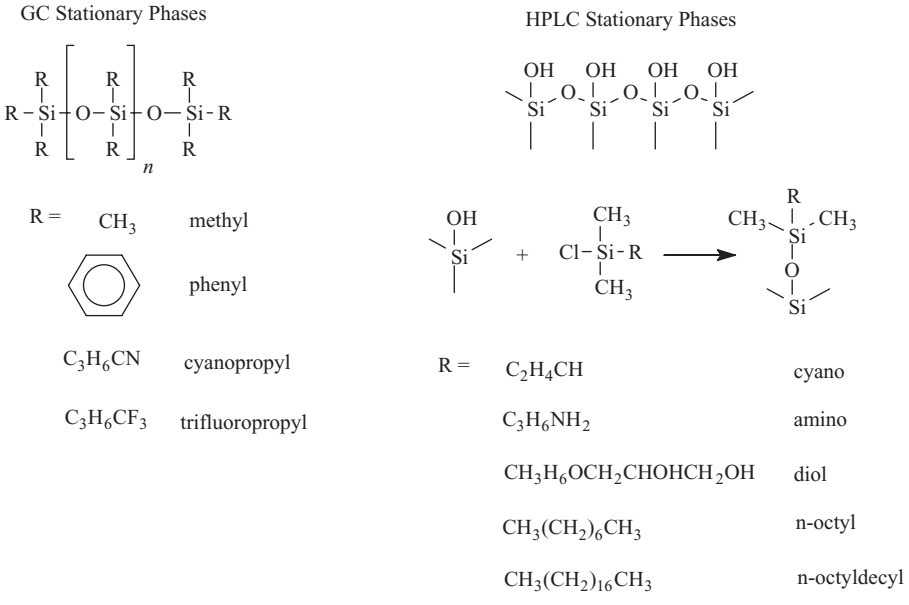


Figure 8.11 Stationary phases found in most GC fused-capillary columns and HPLC columns.

similar, a method that calls for one can usually be easily modified to use the other without having to make too many changes in operating conditions.

Another important decision is the choice of stationary phase or more specifically the type of column to purchase. A detailed discussion of columns is somewhat beyond the scope of this text, but some basic principles will be useful. The stationary phase in GC columns can be nonpolar, semi-polar, or highly polar. The most common stationary phases in fused-silica capillary columns (see Fig. 8.11) have as the core structure the polydimethyl siloxanes where the R-groups are methyl groups. With only methyl groups, the column is nonpolar. To increase the polarity of the column, polar groups such as phenyl, cyanopropyl, or triflouropropyl are substituted for the methyl substituents. The degree of substitution is usually designated by the percentage of substituted methyl groups. For example, a (50%-cyanopropyl)-methylpolysiloxane column has 50% of the methyl groups replaced with cyanopropyl substituents making this a high-polarity stationary phase.

Column length affects the resolution with longer column lengths yielding better separation, but with longer columns, analysis time increases. A nonpolar column essentially separates FAMEs by boiling point. FAMEs with less carbon have lower boiling points, and as the number of double bonds increases the boiling point also goes down. In nonpolar separations of FAMEs of the same length, therefore, the unsaturated species would elute first followed by the saturated component. For example, in a mixture of the 18 carbon FAMEs, α-linolenic would elute first, followed next by linoleic, oleic, and lastly stearic. With polar columns, unsaturated species elute after the parent compound and typically in order of increasing number of double bonds. Another general rule of thumb is that an n6 isomer will elute after its n3 counterpart, for example, 18:3n3 elutes before 18:3n6 on polar columns. With VLCPUFA the order of elution becomes more complicated such as for 22:5n6, 22:6n3, 24:0, and 24:1 (see Fig. 8.12).

On the market today are dozens of fused-silica capillary columns specifically designed to separate FAMEs, and for as many columns as there are, there are as many GC operating conditions.

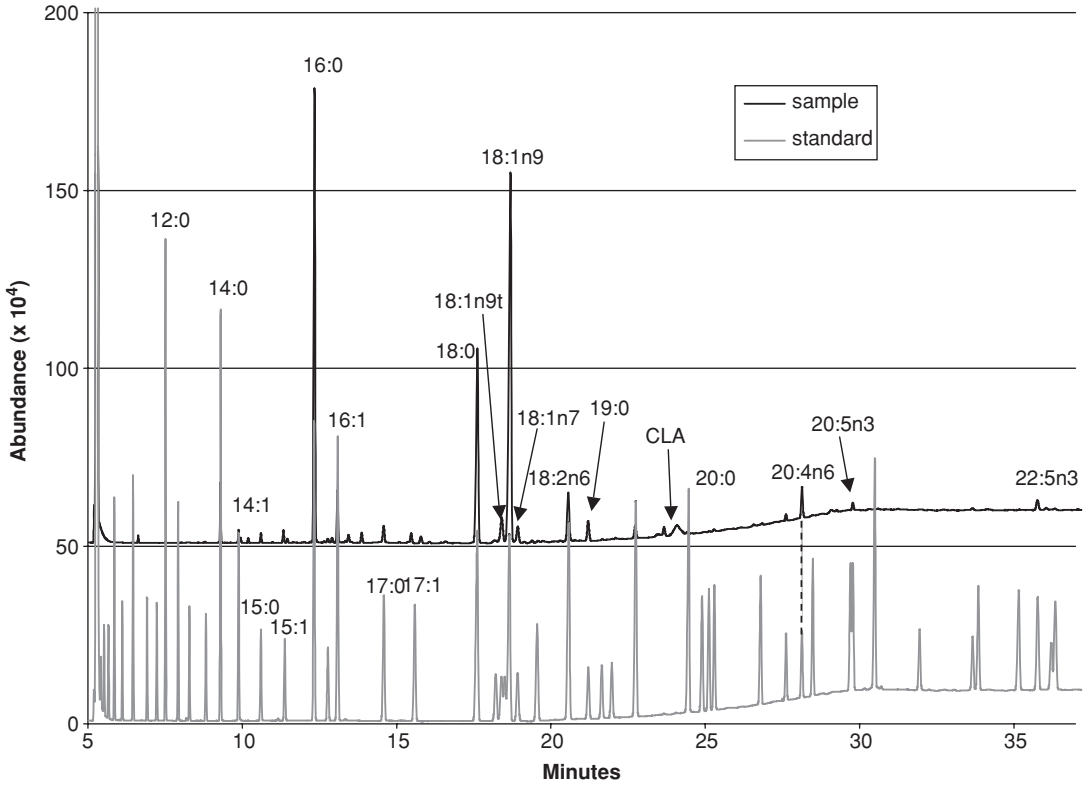


Figure 8.12 Overlaid gas chromatograms of a FAME standard (GLC-463) and beef.

Conditions that are usually required to be known by the investigator are the inlet flow pressure, split ratio, injection temperature, column temperature, column mobile phase flow rate, temperature program, and the FID temperature. Most manufacturers will provide a sample chromatogram of a separation of a FAME standard using their column that includes GC temperature conditions and flow rates, etc.

The column that has been used for several years now in the author's laboratory is the DB-23, 60 m column (J&W Scientific/Agilent Technologies, Santa Clara, CA, USA). A typical chromatogram of a standard FAME mixture, GLC-463, using this column is shown in Figure 8.12. Several of the peaks have been labeled. In order to obtain a fatty acid profile, a wt% of total fatty acids is appropriate. The area under each peak is determined (usually done by GC operating software) and then the total area is summed up. Each individual peak area is next divided by the total area and multiplied by 100 to give a percent contribution of that component to the total. For example, in the sample chromatogram in Figure 8.12 the total area under all of the individual peaks, as determined by the instrument software, summed to 2000 units. The area under the largest peak, 18:1n9, was 650 units. Dividing 650 units by 2000 units and multiplying by 100 gives 32.5%, that is, 32.5% of all the total identified FAMEs consisted of methyl oleate.

Figure 8.12 illustrates two important aspects of GC and the FID: (1) the response of the detector is not the same over the entire range of FAMEs, and (2) peaks broaden and become shorter for those analytes that take longer to elute from the column. For example, the FAMEs 12:0, 14:0, 16:0,

18:0, and 20:0 have all been added at the same amount in the GLC-463 standard. The height or intensity of the peaks corresponding to these FAMEs on the standard chromatogram, however, is different; 12:0 is the tallest followed by then 14:0, 16:0, 18:0, and 20:0. In contrast, the peak areas are 243, 250, 255, 257, and 259 for 12:0, 14:0, 16:0, 18:0, and 20:0, respectively (once again, these are the peak areas that came from the GC operating software). The response of the FID is greater for the longer-chain (bigger) molecules. Over this range the difference is relatively small, 243/259, a change of about 6% meaning that signal for 20:0 is 6% greater than for 12:0. To adjust for this, response factors can be used. The response of the 18:0 peak is assumed to be 1 and the other FAME responses are set to this. The calculation below shows how this can be used for the 14:0 response and how to correct for it in order to obtain a corrected wt% for the amount 14:0 in the sample chromatogram:

$$\text{for 18:0, response factor} = \frac{257}{257} = 1$$

$$\text{for 14:0, response factor} = \frac{250}{257} = 0.973$$

$$\therefore \text{adjusted wt\% for 14:0}$$

$$\frac{\text{area 14:0}}{\text{total area}} \times \text{response factor} = \frac{250}{2000} \times 0.973 = 12.2\%$$

Theoretical response factors have been tabulated and can be used directly, but the researcher is encouraged to obtain response factors specific to their GC system.

The other most common way to report fatty acids is to do so on an mg/100 g of tissue basis done by adding an internal standard, typically 13:0, 19:0, or 23:0 as these fatty acids are rarely found in meat samples. The standard can be added as the methyl ester, as a triacylglycerol, or as the phosphoplipid. A good internal standard is compound that mimics the other analytes of interest, behaves the same as the other analytes, but does interfere with the other analytes. Since most of the fatty acids in meat samples will be in the form of triacylglycerols or phospholipids such as PC, it is best to use these as opposed to a methyl ester. In the sample chromatogram in Figure 8.12, 1.0 mg of trinondecanoin (triacylglycerol with three esterified 19:0 fatty acids) was added into the sample before extraction and transesterification. The assumption using an internal standard is that loss of analytes will be the same to both the sample and standard. The area of the 19:0 FAME peak was 10 units which is to say that 1.0 mg of nondecanoate gives a signal of 10 units. The area of the 18:2n6 peak is 45 units which corresponds to 4.5 mg, and if this was obtained from a 1.0 g sample of meat, then the amount would typically be recorded as 45 mg/100 g of meat.

$$\frac{\text{mg 19:0}}{\text{area 19:0}} = \frac{\text{mg 18:2n6}}{\text{area 18:2n6}}$$

$$\therefore \text{area 18:2n6} \times \frac{\text{mg 19:0}}{\text{area 19:0}} = 45 \times \frac{1.0\,\text{mg}}{10} = 4.5\,\text{mg}$$

$$\text{and } \frac{4.5\,\text{mg}}{1.0\,\text{g meat}} \times \frac{100}{100} = \frac{45\,\text{mg}}{100\,\text{g}}$$

Once again, this calculation can be corrected by multiplying the areas of the peaks by the correct response factors as well.

Before ending the discussion of FAMEs and GC, it should be noted that GC can also be used to quantify other lipids and methods exist for determination of vitamins E and A and for cholesterol but these methods will not be addressed here.

High-Performance Liquid Chromatography

HPLC is typically used to separate the nonsaponifiable lipids and just as with GC the investigator needs to make a decision between a polar or nonpolar column. For historical reasons, nonpolar HPLC is referred to as normal-phase high-performance liquid chromatography (NP-HPLC), while polar HPLC is called reverse-phase high-performance liquid chromatography (RP-HPLC). In NP-HPLC, nonpolar solvents are used for the mobile phase and in RP-HPLC polar solvents are used. The core matrix of the stationary phase in HPLC columns consists of silica beads usually having diameters of 3, 5, or 10 μM. Chemically reactive silanol groups are spread about on the beads (Fig. 8.11) with a surface density of ~8 μmol/m^2 of polar hydroxyl groups. To these silanol groups, various siloxanes are introduced by reaction with organochlorosilanes. The R-group in Figure 8.11 can be any number of different functional groups in order to adjust the polarity of stationary phase. For example, cyano, diol, and amino groups can be added for polar phases, while for most nonpolar phases long alkyl groups such as *n*-octyl (8 carbons), *n*-octyldecyl (18 carbons), and even *n*-tricontanyl (30 carbons) are added. These nonpolar columns are referred to as C8, C18, etc., columns.

Methods of chromatography for the analysis of lipid-soluble vitamins and carotenoids have been reviewed and the majority of these lipid components are separated using RP-HPLC (Barua et al., 2000; Nelis et al., 2000). In particular, RP-HPLC lends itself to concurrent analysis of vitamin E, vitamin A, and carotenoids. RP-HPLC columns also tend to give more consistent retention times, are easier to equilibrate, and are more stable than NP-HPLC columns. Thus, the discussion from this point will be restricted to RP-HPLC.

For separation of the vitamins A and E and some carotenoids, a C18 column is usually used; however, a C8 column will often provide adequate separation. If complete resolution of all geometric (*cis*, *trans*) and positional isomers of some carotenoids (e.g., lycopene) is required, however, a C30 column must be used. The mobile phases typically used consist of acetonitrile, methanol (or some other alcohol), either separately or in binary or trinary mixtures. Occasionally, tetrahydrofuran, dichloromethane, or an electrolyte (salt) solution is added to the mobile phase.

Lipid Oxidation in Muscle Foods

Basic Chemistry of Lipid Oxidation

Molecular oxygen is a somewhat unique diatomic homonuclear molecule in that it can be found in either a singlet or a triplet spin state. Understanding spin state requires a brief review of atomic and molecular orbital theory. Figure 8.13 shows the molecular orbital diagrams for triplet oxygen, $^3O_2$, and singlet oxygen, $^1O_2$. Electrons are placed into available orbitals starting with the lowest energy orbital and working up. Each orbital can contain two electrons and these electrons must be paired with opposite spins; $+1/2$ for spin up, ↑, and $-1/2$ for spin down, ↓. Electrons must have opposite spins because of the Pauli's exclusion principle, which states that no two electrons can have identical quantum numbers. If two electrons are in the same orbital, say, the 2s orbital, then they have the same principal quantum number, $n = 2$, angular momentum number, $l = 0$, and magnetic quantum

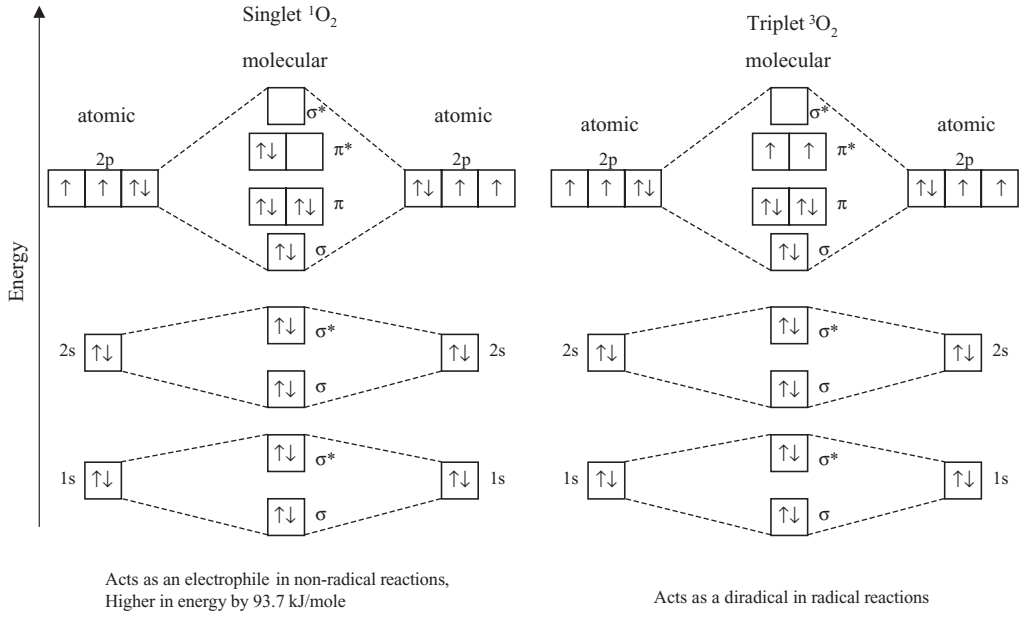


Figure 8.13 Molecular orbital diagram of oxygen.

number, $m_l = 0$. Therefore, the electrons must have opposite electron spin quantum numbers, $m_s = +1/2$ and $-1/2$. The spin state of an atomic or molecular species is defined by the spin multiplicity rule, $2S + 1$, where S is the total spin quantum number achieved by adding the spin quantum numbers of the two highest-energy electrons. If two electrons are paired in the same orbital, their spins are opposite, thus $S = 0$, and the total spin state is 1 (singlet). If the two electrons occupy adjacent orbitals, then because of Hund's rule, the spin state of electrons are the same and $S = 1$ and total spin state is 3 (triplet). Singlet oxygen violates Hund's rule whereby the lowest energy state is achieved when electrons are placed into the orbitals of a subshell with spins parallel whereby one electron is placed into each isoenergetic orbitals before a second electron is added. Singlet oxygen is therefore highly unstable and reactive with an energy of 94 kJ/mole, higher than that of triplet oxygen (Kim and Min, 2008).

Triplet oxygen behaves as a diradical since it has two unfilled orbitals while singlet oxygen is a strong electrophile. A lipid molecule (an unsaturated fatty acid) is in a singlet ground state while molecular oxygen is in a triplet state. A singlet–triplet interaction leading to a reaction is highly improbable and thus most lipid oxidation is caused when oxygen is activated or due to the presence of reactive oxygen species (ROS). Activated oxygen is molecular oxygen in its singlet state which can react directly with an unsaturated fatty acid, while ROS includes such compounds as the hydroxyl radical, •OH; superoxide, $\bullet O_2^-$; hypochlorus acid, HOCl; and hydrogen peroxide, H_2O_2.

Figure 8.14 shows the basic process of fatty acid oxidation. An esterified linoleic acid moiety is used as an example as it is one of the most common PUFA found in phospholipids. Removal of a hydrogen and electron occurs at the methylene between the two double bonds (an allyl position as shown by the *star*) since it requires the least energy to do so, ~200 kJ/mole (Kim and Min, 2008). Other potential hydrogen abstraction positions, the methyl on the end or other interior methylene positions (indicated by the *arrows*), require more energy to remove the hydrogen, ~300 and

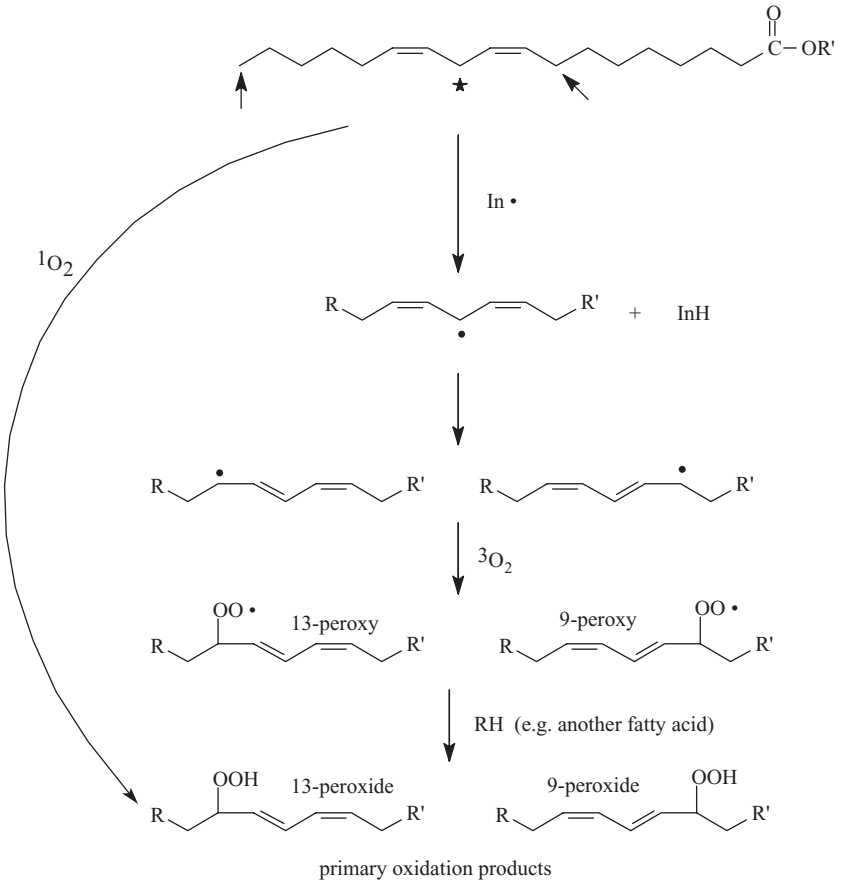


Figure 8.14 Oxidation of a fatty acid (e.g., linoleic acid) ending primary oxidation products.

400 kJ/mole, respectively. Abstraction occurs by an initiator such as any available radical present, the most likely being a hydroxy radical, •OH. The radical fatty acid readily rearranges to a conjugated radical species that then reacts with $^3O_2$ resulting in the peroxy radical. Propagation can now occur where the peroxy radical abstracts a hydrogen atom from an available species, in many instances this is another fatty acid creating another radical fatty acid moiety. The fatty acid peroxide is relatively stable and it is referred to as a primary oxidation product. Although somewhat stable, a variety of factors present in meat can cause further reactions resulting in the fatty acid hydroxyl radical. Fatty acid hydroxyl radicals eventually decompose into such species as ketones, aldehydes, hydrocarbons, and alcohols as shown in Figure 8.15. Obviously, the situation is different with a MUFA such as oleic acid, or a highly unsaturated fatty acid such as α-linolenic, or VLCPUFAs such as n3 and n6 C20, C22 fatty acids. Initial hydrogen abstraction would not occur as easily in a monounsaturated molecule due to higher strength of CH bonds as mentioned above, while in a highly unsaturated species, their oxidation would occur more readily as there are more allyl methyl carbons. In Figure 8.15 only a few secondary products are shown in order to give the reader an idea of the types of secondary molecules produced, but there are dozens and dozens of potential secondary products from the many types of PUFAs.

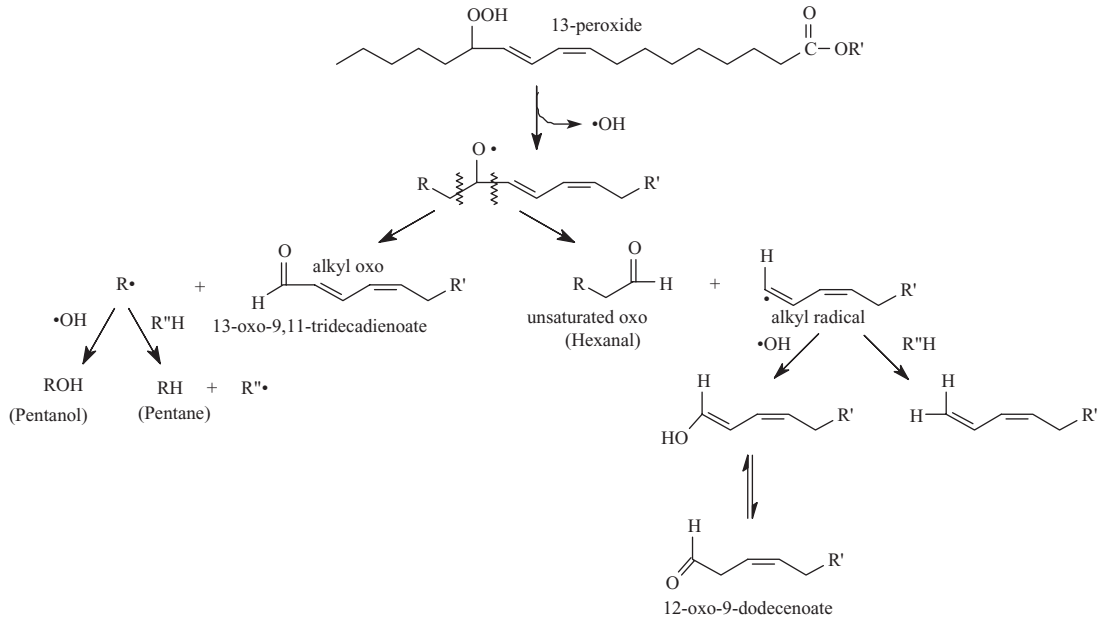


Figure 8.15 Secondary oxidation products from a peroxide fatty acid (a primary product).

Initial hydrogen abstraction can also be caused by sensitizers. A sensitizer is a molecule that absorbs visible or ultraviolet electromagnetic radiation and transfers its energy to triplet oxygen creating singlet oxygen leaving the sensitizer in an excited triplet state (Fig. 8.16). Singlet oxygen can be formed due to enzyme activity, chemically, and most importantly in foods photochemically by sensitizers such as chlorophyll, porphorins, riboflavin, myoglobin, and color additives. Triplet excited sensitizers easily start the initiation of fatty acid oxidation. It is important to note also, however, that as shown in Figure 8.14, singlet oxygen can react directly with the fatty acid moiety to directly produce the peroxide. The reaction of singlet oxygen with unsaturated fatty acids is 1500

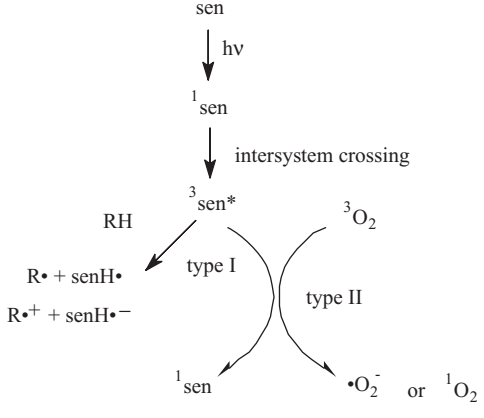


Figure 8.16 Activation of triplet oxygen by a sensitizer to produce either superoxide or singlet oxygen.

times faster than with triplet oxygen (recall direct reaction of triplet oxygen with a fatty acid is spin forbidden) (Frankel, 1998).

Enzyme and Nonenzyme Reactive Oxygen Species

The ROS that help initiate and propagate lipid oxidation can arise due to a number of factors. Several of the important nonenzymatic chemical reactions involve the cleavage of hydrogen peroxide, H_2O_2, into the highly reactive hydroxy radical, $\bullet OH$, and hydroxide anion. Heat and light can cleave hydrogen peroxide directly:

$$H_2O_2 \xrightarrow{\Delta \text{ or } h\nu} \bullet OH + OH^-$$

metal cations in particular iron (II):

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + \bullet OH + OH^-$$

and superoxide.

$$H_2O_2 + \bullet O_2^- \rightarrow {}^1O_2 + \bullet OH + OH^-$$

Certain post-mortem changes in the animal tissue can also affect oxidation such as loss of integrity of cellular phospholipids membranes. Organelles such as lysosomes, therefore, can release their enzymes such as phospholipases and lipases. These enzymes cleave fatty acid moieties from phospholipids and triacylglycerols further affecting membrane integrity and freeing fatty acids for further oxidation. Other enzymes that are released or are naturally occurring in muscle tissue such as lipoxygenase, myeloperoxidase, and cycloxygenase can act as catalysts in other degradative processes.

Lipoxygenase is an iron-containing dioxygenase enzyme, that is, an enzyme that adds both atoms of an oxygen molecule into another compound. In the case of lipoxygenase, an example of the reaction with linoleic acid is:

$$\text{Linoleate} + O_2 \rightarrow \text{13-hydroperoxyoctadeca-9-11-dienoate.}$$

Cyclo-oxygenase can act as either a dioxygenease (like reaction above with linoleate) or as a peroxidase:

$$ROOR' + 2e^- + 2H^+ \rightarrow ROH + R'OH.$$

The reactions associated with myeloperoxidase are:

$$H_2O_2 + Cl^- \rightarrow HOCl + OH^-$$
$$HOCl + \bullet O_2^- \rightarrow O_2 + Cl^- + \bullet OH$$

producing the hydroxy radical.

Another initiator or propagator of oxidation is heme-bound iron cations. For example, metmyoglobin, MbFe(III), can react with peroxides, either H_2O_2 or ROOH species, producing an unstable perferrylmyoglobin radical that converts readily to the stable ferrylmyoglobin.

$$\text{MbFe(III)} + \text{ROOH} \rightarrow \text{ROH} + \bullet\text{MbFe(IV)O}$$
$$\bullet\text{MbFe(IV)O} + \text{ROOH} \rightarrow \text{MbFe(IV)O} + \text{ROO}\bullet + \text{H}^+$$

Free iron(II) also plays a role in initiation of propagation of oxidation. The free iron cations are present in small amounts initially, 2–5% of total iron in the myocyte, but it can be released from heme and other proteins such as lipoxygenases as the tissue and membranes degrade. Cationic iron can convert singlet oxygen to the reactive superoxide species,

$$^1\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \bullet\text{O}_2^-$$

or activate hydrogen peroxide.

Measurement of Oxidation

There are several methods useful for measuring lipid oxidation. Methods to assess primary oxidation products measure the level of peroxides and conjugated dienes (refer to Fig. 8.14), while measurements of secondary products assess carbonyl compounds (the anisidine test), aldehydes (thiobarbituric acid test), or a combination of these volatile compounds (see Fig. 8.15) using GC or by human sensory panels. These methods have been ranked (Frankel, 1998) in order of their usefulness. The methods in order of most to least useful are: sensory evaluation, measurement of volatile products by GC, UV absorbance due to conjugated dienes, measurements of carbonyls and anisidine values, peroxide value (PV), oxygen absorption, thiobarbituric acid, carotene bleaching, and measurement of volatile acids by the Rancimat apparatus. Not all of these will be discussed here but the most useful or commonly used methods will be presented.

Volatile Products, Sensory Evaluation, and Headspace Gas Chromatography

The oxidized products of lipids can have specific effects on unwanted flavors in meat. Off-flavors in meat such as fishy, oily, and grassy flavors are due to the volatile compounds produced during oxidation, mostly the aldehydes (Kim and Min, 2008). These aldehydes have threshold values in parts per trillion. The threshold value is the lowest concentration at which 50% of subjects in a sensory evaluation can detect the presence of a compound. Since these volatile compounds have the most impact on flavor, it is useful to correlate sensory evaluation with measurement of these volatiles using GC. Sensory evaluation is perhaps the most useful measure of oxidation since taste and flavor are the most important components for consumer acceptance (Frankel, 1998). Sensory evaluation is, however, the least reproducible and, for obvious logistical reasons, the most difficult. It is usually done with a trained panel of 5 to 25 subjects called an analytical panel, or a consumer panel consisting of 50 or more untrained subjects. Analytical panelists are trained to rank samples in reference to known standards while consumer panel will simply make judgments based on acceptability or preference.

Primary Changes

The primary or initial changes during oxidation would be the loss of reactants such as the fatty acids, and a gain in products, namely hydroperoxides. Loss of fatty acids or change in fatty acid profile has been used but direct measure of the hydroperoxides is most common. Initially, the amount of hydroperoxides formed is greater than their rate of degradation, and there is a growth period and decay period for hydroperoxides making this method difficult in that the analytes are unstable and transient.

One means of measuring hydroperoxides is to measure the PV. The PV can be obtained by the reduction of the hydroperoxides with the iodide anion followed by back titration of molecular iodine with thiosulfate (this is the AOAC method).

$$\mathrm{ROOH} + 2\mathrm{H}^+ + \mathrm{KI} \rightarrow \mathrm{I}_2 + \mathrm{ROH} + \mathrm{H}_2\mathrm{O} + 2\mathrm{K}^+$$
$$\mathrm{I}_2 + 2\mathrm{Na}_2\mathrm{S}_2\mathrm{O}_3 \rightarrow \mathrm{Na}_2\mathrm{S}_4\mathrm{O}_6 + 2\mathrm{NaI}$$

This is in fact the AOAC method (9565.33) but is, however, time-consuming. Other methods have been developed such as ones based on the oxidation of Fe^{2+} to Fe^{3+} (Shantha and Decker, 1994).

The conjugated diene and triene systems which result from hydroperoxide formation can be measured easily by ultraviolet absorption at 234 nm (dienes) and 268 nm (trienes). After extraction of the lipids, the extract can be subjected to this photometric assessment directly. Measurement of conjugated hydroperoxides is therefore significantly easier than the iodometric methods but if there are other molecules with conjugated systems, such as carotenoids, then their interference can be considerable.

Secondary Changes

Secondary changes are usually more appropriate for the measurement of oxidation in foods because hydroperoxides are transient, colorless, and flavorless, while secondary products such as ketones, aldehydes, hydrocarbons, and alcohols are often quite odorous and form stable albeit volatile end-products.

The single most common means of measuring secondary products is by the reaction of 2-thiobarbituric acid (TBA) with aldehydes (Fernández et al., 1997). The so-called thiobarbituric acid reactive substances (TBARS) test has been used for decades (Bernheim et al., 1948) and although it has many limitations, it still serves as a benchmark for any measurement of lipid oxidation. The end-products of lipid hydroperoxides (see Fig. 8.15) are the secondary products such as hexanal, pentanal, 4-hydroxynonenal, and malondialdehyde (MDA). MDA is used as the standard in a TBARS measurement and as such values are typically reported in units of MDA equivalents. The reaction is shown in Figure 8.17 and the resulting product, the TBA-MDA adduct results in a colored product that can be measured photometrically at 532 nm. The TBARS test is usually conducted by reaction of the TBA solution with either (1) the meat product itself, (2) an aqueous extract or a steam distillate of the meat sample, (3) with a lipid extract from the meat (Fernández et al., 1997).

One limitation to the TBARS test is that the rate of the reaction can be altered by changes in pH, temperature, and concentration of the TBA (Fernández et al., 1997). Lower pH, higher temperatures, and/or higher TBA concentrations will increase the rate of the reaction. It is important, therefore, to maintain consistency in assay condition. Another limitation is that the aldehydes are somewhat unstable and transient and are subject to further oxidation to alcohols and acids.

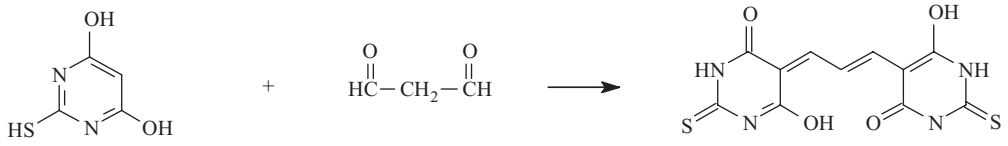


Figure 8.17 The thiobarbituric acid test (TBARS). The reaction between malonaldahyde and 2-thiobarbituric acid is shown which results in the colored ($\lambda = 530$–532 nm) TBA–MA adduct.

Thus, the potential exists that the levels of MDA and other aldehydes will change due to the reaction conditions. Further, the degree to which TBA reacts with other aldehydes is not well known. There are also a number of other species in matrix that can interfere with the MDA-TBA adduct (Fernández et al., 1997). The researcher needs to investigate the various methods thoroughly before proceeding with TBARS test and must realize that comparisons of MDA equivalents among various studies from other investigators are highly questionable. Within an individual investigation, however, the measurement of MDA equivalents is very useful to measure the overall amount of oxidation, that is, interstudy comparisons should be avoided, but intrastudy comparisons are acceptable.

Procedures

TBARS

This is a simple modified method based on the procedure given by Wang et al. (2002). A 5 g (weighed to the nearest 0.01 g) sample of meat is homogenized in 40 mL of homogenizing solution (1000 mL solution of 7.5% trichloroacetic acid, 0.1% propyl gallate, and 0.1% EDTA) for 5 min. The homogenate is diluted with 10 mL more of the homogenizing solution and then centrifuged for 5 min at 6000 rpm.

An aliquot of 2.00 mL of the supernatant is then mixed with 2.00 mL of 80 mM solution of TBA solution. The mixture of supernatant and TBA solution is then incubated for 90 min in a water bath at 40°C. Absorbance of the TBA adducts is measured using a UV–vis spectrophotometer at 532 nm.

Folch Total Lipids Extraction

1. Chop finely a 0.5–2.0 g sample (weighed to the nearest 0.0001 g) and place into a beaker or flask (50 mL flask works best).
2. Add to this sample 1.0–2.0 mg of C19 PC standard.
3. Add 20–40 mL of a 2:1CM mixture and homogenize under nitrogen in an ice bath (2–4 min at setting 4–5).
4. Filter the homogenate through filter paper (Whatman #4, i.e., qualitative grade) into a 50 mL graduated cylinder.
5. Split the filtered sample in half and add to each aliquot 0.1 M NaCl solution. The sodium chloride solution needs to be added in a 4:1 ratio with the filtered sample solution. For

example, if 30 mL of filtered solution is collected, then put 15 mL into two new test tubes. Add to each of these two samples 3.75 mL of 0.1 M NaCl.

$$\text{e.g.}\quad \frac{4}{\underbrace{15\text{ mL}}_{chlorform;methanol}} = \frac{1}{\underbrace{x\text{ mL}}_{0.1M\ \text{NaCl}}}$$
$$x = 3.75\text{ mL}$$

6. Flush the sample with nitrogen and vortex for at least 3 min.
7. Centrifuge the sample at 2000 rpm for 5 min.
8. The top layer is the aqueous layer; it can be discarded. The bottom layer is the organic layer.
9. Add 2 mL of upper-phase solution (chloroform:methanol:water at a ratio of 3:48:47; v/v) to the remaining organic bottom layer and gently rotate the solution by hand. This step is helping to wash out any remaining nonlipid components. The mixture was allowed to settle and the remaining top aqueous layer was once again removed.
10. The bottom organic layer contains the lipids. The solvent can be dried off using a nitrogen evaporator.
11. Once dry, the sample (it should look like a small yellowish oily smudge at the bottom of the tube) can either be reconstituted into 1 mL DCM and stored in the freezer, or methylated.

Notes

- The methanol has BHT added to it, 50 mg/L.
- Always flush out test tubes that contain sample with nitrogen before capping them when storing, vortexing, heating, etc. Use only the Teflon (PTFE) lined caps with glass tubes.

Direct Transesterification (Methylation) of Fatty Acids

This procedure is directed by O'Fallon et al. (2007).

1. Add a 1 g sample (weighed to the nearest 0.001 g) of meat to a test tube containing 80 μL of the C19:0 internal standard, 0.7 mL of 5 M KOH solution, and 5.3 mL of MeOH.
2. Incubate the sample at 55°C in a water bath for 90 min with constant shaking.
3. Remove the sample and allow it to cool to room temperature and then add 0.58 mL of 12 M H_2SO_4.
4. Mix the sample and incubate again in the water bath at 55°C for 90 min with constant shaking.
5. Cool the sample in a cold tap water bath and then add 3 mL of hexane.
6. Mix the sample briefly by inversion and then vortex mix for 5 min.
7. Centrifuge for 5 min in a tabletop centrifuge and collect the top (hexane) layer which contains the FAME and place it into a GC vial. The FAME sample can be stored at −20°C until analysis.

Nonsaponifiable (Cholesterol, Fat-Soluble Vitamins) Standard Solutions

α-Tocopherol (400 μg/mL) and cholesterol (2 mg/mL) stock solutions were prepared daily in hexanes. Individual solutions for α-tocopherol were prepared daily by diluting the stock solution to

cover the concentration range of 0.04–80 μg/mL. Individual solutions for cholesterol were prepared every two weeks to cover the concentration range of 0.03–2 mg/mL. Solutions were protected from light and kept at -20^{O}C when not in use. 5α-cholestane was prepared by dissolving 20 mg/mL in hexanes. Ethanolic KOH solution was prepared daily by dissolving 11% w/v of KOH in 55% ethanol and 45% water.

Extraction and Sample Preparation for Nonsaponifiable Lipids: Cholesterol, Fat-Soluble Vitamins, Carotenoids

This is essentially a modified procedure based on Liu et al. (1996). An approximate 1 g (weighed to the nearest 0.1 mg) thawed sample of meat is chopped finely and placed in a 15 mL Pyrex tube. Vitamin C (0.25 mg), 250 μL of 5α-cholestane, and approximately 7.3 mL of digestion solution are added to the meat sample. The mixture is vortex mixed for 10 s or until all vitamin C is dissolved. The meat sample is then digested in a water bath (80°C) with regular shaking for 15 min or until all the meat samples are fully digested. The sample is cooled immediately and 4 mL of hexane is added, vortexed for 2 min, and then cooled in ice for 5 min. The upper phase is collected and filtered through 0.20 membrane filter (Millipore filters). This filtered upper phase is then evaporated using a N_2 evaporator to dry off the organic layer. The dried sample is reconstituted with 250 μL hexane.

Gas Chromatography (GC)

The FAMEs determination using capillary GC:

The author currently uses an Agilent 6890N Network Gas Chromatography system equipped with a FID, an Agilent 7683 series auto sampler, and a J&W DB23 (50% cyanopropyl-methylpolysiloxane) column (60 m × 0.320 mm, ID, 0.25 μm film thickness). Helium is used for the carrier gas with a flow rate at 2.0 mL/min. An injection split ratio of 25:1 is used and the injector and detector temperatures are set at 280°C. The oven temperature program used is:

1. initial temperature of 130°C which is held for 1 min;
2. increase to 170°C at a rate of 6.50°C/min;
3. another increase to 215°C at a rate of 2.75°C/min and a held for 12 min;
4. final increase to 230°C at 40.00°C/min and held for 3 min.

High-Performance Liquid Chromatography (HPLC)

The author currently uses an Agilent 1100 Series HPLC system with a Varian C18 reverse-phase column (4.6 mm × 100 mm, 3 μm particle size). Two solvents are used for the mobile phase. Solvent A composed of methanol–water (3:1, v/v) containing 10 mM ammonium acetate and Solvent B is methanol–dichloromethane (4:1, v/v) mixture. A flow rate of 0.8 mL/min is used. A linear gradient from Solvent A to Solvent B over a 20 min period followed by 100% Solvent B for 20 min. Then, a reverse linear gradient over 5 min from 100% Solvent B to 100% Solvent A. The column was then allowed to equilibrate for 10 min with Solvent A. The injection volume was 20 μL, and the column temperature was uncontrolled. The variable wavelength detector was set at 290 nm, 340 nm, and 445 nm. The run time for each sample was 55.0 min.

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9 Meat Color

Richard Mancini

Introduction

Color significantly influences meat purchasing decisions because consumers use discoloration as an indicator of product spoilage and wholesomeness. This can result in an annual revenue loss of $1 billion to the beef industry (Smith et al., 2000). Recovery of lost profit via improved color stability relies on our understanding of the fundamental principles associated with myoglobin redox chemistry.

Meat Color Chemistry

Myoglobin is a sarcoplasmic protein that determines meat color via its centrally located heme iron. This globular single-chain protein consists of 153 amino acid residues contained within 8 α-helices joined by short nonhelical strands. Two histidines (64 and 93) interact with myoglobin's iron-containing protoporphyrin heme to effect protein structure, functionality, and meat color stability.

Iron within the heme ring has the ability to form six bonds. Four of these bonds connect iron to the protoporphyrin via coordination with pyrrole nitrogens. The 5th bond links the prosthetic heme group to the apoprotein via coordination with the proximal histidine (also referred to as H93 or F8). The 6th coordination site on iron is able to reversibly as well as preferentially bind ligands such as oxygen, carbon monoxide, and nitric oxide. The ligand present at this 6th coordination site and the redox state of iron determine meat color via the production of four possible chemical forms of myoglobin (deoxymyoglobin, oxymyoglobin, carboxymyoglobin, and metmyoglobin) (Figs. 9.1–9.3).

Deoxymyoglobin

Deoxymyoglobin produces a purplish-red color, resulting from a combination of ferrous heme iron (Fe^{2+}) and an unoccupied (no ligand present) 6th coordination site. As the name implies, this myoglobin form is associated with muscle foods that are not exposed to oxygen (deoxy) and can be found either in vacuum-packaged meat or within the interior of freshly cut meat.

The Science of Meat Quality, First Edition. Edited by Chris R. Kerth.

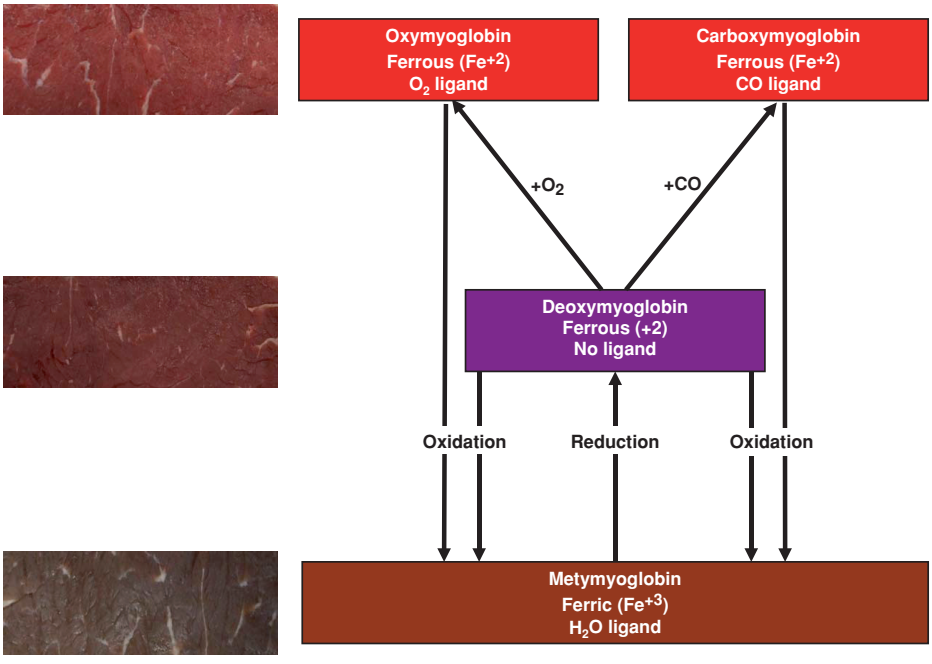


Figure 9.1 The effects of myoglobin redox state, ligands, and iron valence on the surface color of postmortem muscle. For a color version, see plate 9.1.

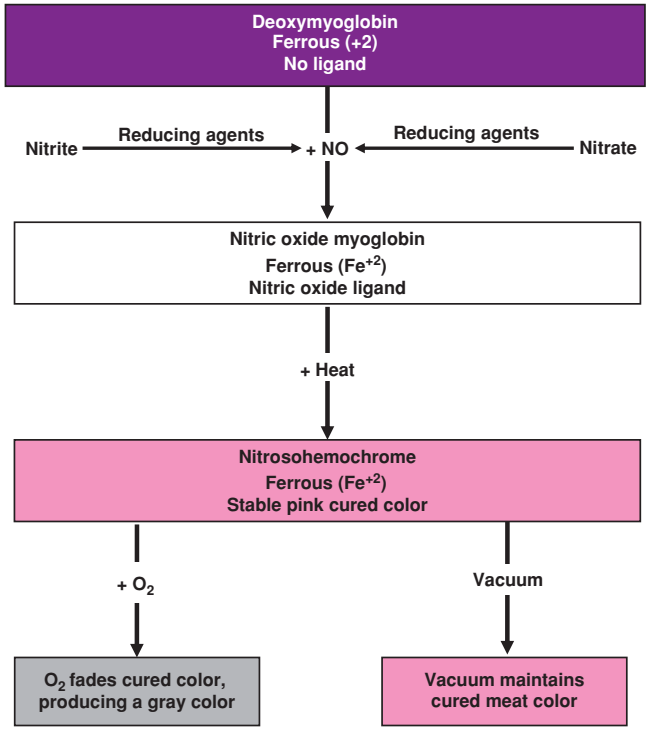
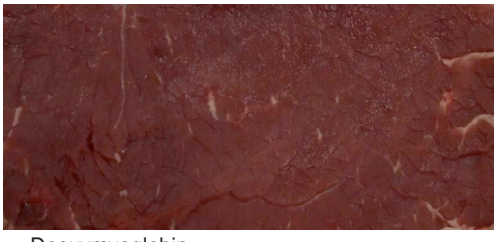


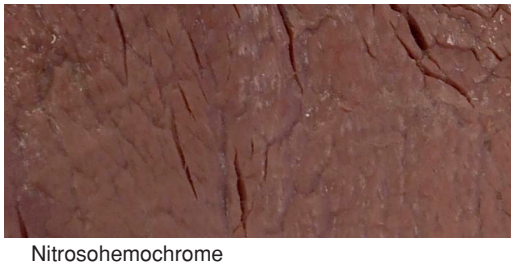
Figure 9.2 Cured color formation and fade. For a color version, see plate 9.2.



Deoxymyoglobin

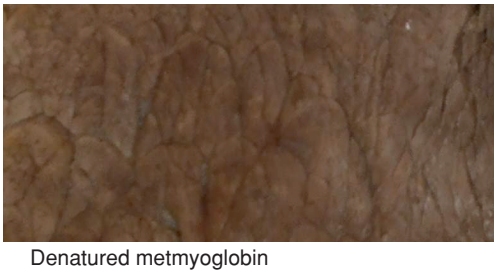
Nitric oxide myoglobin

Oxymyoglobin

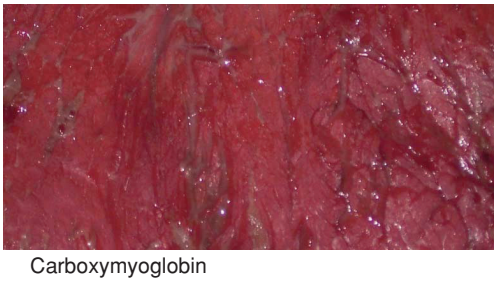


Nitrosohemochrome

Metmyoglobin



Denatured metmyoglobin



Carboxymyoglobin

Figure 9.3 Colors associated with myoglobin forms on the surface of beef. For a color version, see plate 9.3.

Oxymyoglobin

Oxygenation results when deoxymyoglobin is exposed to oxygen and is necessary for the development of both oxymyoglobin and a bright cherry-red color, a process commonly referred to as bloom. It is important to note that oxygenation has no effect on iron's valence state, but rather creates a situation where oxymyoglobin's 6th coordination site on iron becomes occupied by diatomic oxygen. As a result, oxymyoglobin remains in a ferrous redox state.

Carboxymyoglobin

Carbon monoxide binds strongly to the 6th coordination site of heme iron in deoxymyoglobin to form a bright cherry-red pigment, carboxymyoglobin. It is important to note that the reaction characteristics of carbon monoxide with myoglobin, and the interconversion(s) between carboxy- and other myoglobin forms are poorly understood in relation to meat color chemistry. In particular, myoglobin's tendency to release carbon monoxide and subsequently bind oxygen once carboxymyoglobin is exposed to the atmosphere is a fundamental mechanism that should be reviewed. Moreover, the likelihood of converting oxy- to carboxymyoglobin via replacement of an oxygen ligand with carbon monoxide should receive future attention as this is relevant to packaging previously bloomed steaks in carbon monoxide.

Metmyoglobin

Oxidation of heme iron within either deoxy- or oxymyoglobin produces brown discoloration as a result of metmyoglobin formation. It is important to note that metmyoglobin on the surface of meat products often migrates outward from the interior. As atmospheric oxygen diffuses into meat products, the partial pressure of oxygen decreases as the depth beneath the surface increases. Thus, the interior portion of a steak or roast may be deoxygenated and purple in color even though the surface contains bright-red oxymyoglobin. A layer of brown metmyoglobin forms between the red surface and the purple interior as the result of low-oxygen partial pressure (low meaning not great enough for oxygenation and not anaerobic enough for deoxygenation). This metmyoglobin will eventually shift toward the surface, where it is visible to consumers. The migration of discoloration depends on several factors including packaging, temperature, pH, postmortem age, reducing activity, and microbial growth.

Oxygen Consumption

Competition for oxygen in postmortem muscle, primarily between mitochondria and myoglobin, is the principal determinant of oxymyoglobin formation and thus, the development of bright cherry-red color. More specifically, oxygen consumption by mitochondria will limit myoglobin oxygenation and result in a dark, deoxygenated meat color. This could be due to elevated ultimate pH, which promotes mitochondrial respiration and competition for oxygen in meat. In this situation, meat fails to bloom (oxygenate to a bright cherry-red) and is commonly referred to as dark-cutting beef.

Metmyoglobin Reduction

Myoglobin oxidation produces ferric metmyoglobin and decreases shelf life; thus, maintenance of ferrous deoxy- and oxymyoglobin via chemical reduction of metmyoglobin's ferric iron plays a vital role in meat-color life. Metmyoglobin reducing activity involves the transfer of an electron from a donating source to metmyoglobin. This process can occur by either (1) direct reduction of metmyoglobin via NADH or (2) indirect reduction involving metmyoglobin reductase (a NADH–cytochrome b5 complex). Nevertheless, metmyoglobin reducing activity is dependent on several factors, including oxygen scavenging enzymes, reducing enzyme systems, and the NADH pool, all of which are continually depleted with time postmortem.

Deoxygenation and Subsequent Reoxygenation

Conversion of oxymyoglobin to deoxymyoglobin on the surface of fresh meat is a two-step process where oxymyoglobin is not converted directly to deoxymyoglobin, but first is oxidized by low-oxygen partial pressures. This idea is of particular importance when packaging bloomed product in either vacuum or ultra-low-oxygen atmospheres. In particular, meat must be capable of both oxygen consumption and reduction of ferric to ferrous iron in order to regenerate deoxymyoglobin. From a meat packaging standpoint, this two-step process is often troublesome because deoxymyoglobin formation and subsequent rebloom depend on both reducing capacity and oxygen consumption.

Hemoglobin

The appearance of muscle foods is not only influenced by myoglobin's role in lean color but also by hemoglobin redox state within bone marrow and fat. As a result, the industry has reported that marrow and fat color can affect consumer acceptance. Cutting bone exposes deoxyhemoglobin within marrow to oxygen, producing a bright-red pigment (oxyhemoglobin) that can be oxidized to an undesirable gray or black methemoglobin. The color of subcutaneous fat has been attributed to both the redox state of residual hemoglobin within capillaries and the accumulation of carotenoids.

Antemortem Factors Affecting Meat Color

Antemortem factors can influence meat color (lightness, darkness, and color stability) via several mechanisms, including changes in muscle pH and/or myoglobin redox chemistry. For example, animal diet can affect metabolism, glycogen storage, pH, chilling rate, and antioxidant accumulation, all of which play a role in meat color. This is readily observed when the color of meat from grass-fed and grain-fed cattle is compared.

Pastured animals tend to have less subcutaneous fat than those that are grain-finished. As a result, carcasses from grain-fed animals chill slower and experience warmer muscle temperatures shortly after harvest. This encourages glycolytic enzyme activity, pH decline, protein denaturation, and muscle lightness in grain-finished animals compared with pasture-finished animals (Bruce et al., 2004). Forage-based diets also tend to encourage oxidative metabolism, rather than anaerobic muscle metabolism and glycogen storage (Vestergaard et al., 2000; Baublits et al., 2004). As a result, meat from cattle fed forage-based diets may be darker with a greater ultimate pH, compared with cattle

fed concentrates. Changes in color stability resulting from grass-based diets are primarily attributed to antioxidant accumulation (O'Sullivan et al., 2003).

Pork color is commonly associated with glycolytic potential, which is a measure of muscular capacity for anaerobic metabolism. Research suggests that elevated glycolytic potential encourages acidity and paleness (Meadus and MacInnis, 2000; Juncher et al., 2001; Hamilton et al., 2003; Moeller et al., 2003). Thus, most data indicate that reducing glycolytic potential and free glucose may improve pork muscle color by altering postmortem lactate levels. As a result, strategic finishing of swine using diets low in digestible carbohydrates has been shown to darken loins by reducing muscle glycogen stores (Rosenvold et al., 2001a, 2001b).

Animal activity can play a role in beef color by influencing muscle fiber type and metabolism. For example, increased physical activity can encourage muscle pigmentation and darkness whereas limited activity can decrease the amount of slow-contracting fibers, reduce oxidative metabolism, and increase lactate production (Vestergaard et al., 2000; Wilborn et al., 2004).

Genetics

Genotypes commonly associated with pork color are those related to stress-susceptibility and postmortem pH decline, including the halothane, ryanodine, and napole genes. For example, the halothane genotype influences postmortem muscle pH (nn $<$ Nn $<$ NN), resulting in a lower incidence of pale, soft, and exudative (PSE) meat (8%) in halothane negative pigs (NN) compared with nn genotypes, which can exhibit nearly 100% PSE (Channon et al., 2000; Fisher et al., 2000; Eggert et al., 2002; Fernandez et al., 2002; Moelich et al., 2003). Mutations affecting alleles associated with ryanodine receptors also detrimentally affect lightness, via their involvement in porcine susceptibility to malignant hyperthermia (Bertram et al., 2000; Kuchenmeister et al., 2000; Piedrafita et al., 2001; Van Oeckel et al., 2001; Velarde et al., 2001; Moeller et al., 2003). Although genetic effects on pork color have traditionally been associated with alterations in muscle lightness and paleness, the role of genetics in muscle redness has recently been reported. It is possible that genotypic improvements in redness can result from temperature- and pH-induced effects on oxygen consumption (Lindahl et al., 2004). More specifically, redness and degree of bloom can be enhanced by stress-mediated increases in *longissimus* temperature. Warm muscle temperatures will promote inactivation of proteins involved in oxygen consumption, resulting in greater myoglobin oxygenation (greater degree of bloom) due to less competition for oxygen by enzymes (Rosenvold and Andersen, 2003). However, stress-related elevation of postmortem muscle temperature also can inactivate metmyoglobin reductase and therefore, decrease color stability.

Postmortem Factors Affecting Meat Color

Temperature and Case-Ready Packaging

Cold chain management via maintenance of refrigerated temperatures during both storage and display is critical to food preservation, including meat color and shelf life. Warm temperatures accelerate myoglobin oxidation, lipid oxidation, and microbial growth, all of which will limit shelf life.

Packaging also plays a significant role in meat color. Common types of meat packaging include traditional polyvinyl chloride overwrap, vacuum, and case-ready modified atmospheres. Traditional packaging using Styrofoam trays overwrapped with PVC exposes meat to the atmosphere and allows for the development of bright cherry-red surface oxymyoglobin. Although vacuum packaging can lengthen color life compared with traditional PVC overwrap, the formation of purplish-deoxymyoglobin may negatively influence consumer purchasing. Case-ready packaging using modified atmospheres, such as high-oxygen, ultra-low-oxygen, and carbon monoxide, is also available.

High-oxygen atmospheres typically contain 80% O_2 in order to increase myoglobin oxygenation, depth of oxymyoglobin penetration, and the amount of time before metmyoglobin migrates to the muscle surface. Although high-oxygen atmospheres can improve color life, rancidity resulting from lipid oxidation often develops while color is still desirable (Jayasingh et al., 2002). Conversely, ultra-low-oxygen atmospheres can be used to minimize lipid oxidation. However, inadequate pigment-reducing capacity and limited post-storage surface oxygenation decrease the efficacy of ultra-low-oxygen systems. In addition, the levels of residual oxygen within ultra-low oxygen packages must remain less than 1% for pork and less than 0.05% for beef in order to limit metmyoglobin formation at low partial pressures. Low levels of carbon monoxide can be added to ultra-low oxygen packages because of its ability to improve shelf life via the formation of bright cherry-red carboxymyoglobin (Sørheim et al., 1999; Luno et al., 2000; Jayasingh et al., 2001; Hunt et al., 2004). In general, 0.4–0.5% CO has been effective for improving beef color life (Luno et al., 2000; Jayasingh et al., 2001; Hunt et al., 2004). However, it is important to note that depth of carbon monoxide penetration beneath the meat surface (thickness of the carboxymyoglobin layer) increases as both exposure time and CO concentration increase (Krause et al., 2003). In some cases, carbon monoxide will completely penetrate meat samples and convert interior pigments to carboxymyoglobin.

Central packaging of case-ready meat products also provides the opportunity to use numerous injection-enhancement ingredients that add value and lengthen product shelf life. Injection-enhanced product is fabricated into steaks and packaged in modified atmospheres at a central location; then shipped to retailers who are able to display prepackaged-enhanced product without additional labor associated with cutting and packaging. Enhancement involves adding functional ingredients to whole muscle cuts, including water, salt, phosphate, and lactate. Certain ingredients have the ability to influence both myoglobin and lipid oxidation and thus can affect color life.

Laboratory Analyses of Raw Meat Surface Color

There are several ways to measure meat color and thus selecting the most suitable and efficient method is often not straightforward. Thus, researchers must decide which procedure best matches their particular situation. In general, researchers have two primary options for measuring color: visual and instrumental evaluation. Within each of these categories are two subcategories: consumer and trained panels for visual methodology, and surface reflectance and extraction for instrumental techniques.

The following will outline some of the most commonly used methods for evaluating raw, cooked, and cured meat color, in addition to presenting “Dos” and “Don’ts” regarding color evaluation. Detailed descriptions of color measurement methodology also are available in the Guidelines for

Meat Color Evaluation (AMSA, 1991) and Measurement of Discoloration in Fresh Meat (Faustman and Phillips, 2001).

Regardless of which procedure is used to measure color, it is critical that the experimental conditions match the desired project objective. In particular, researchers must ensure that a representative sample is obtained and that all samples are prepared and analyzed in the same manner. Although lighting can significantly influence visual color evaluations, it is commonly overlooked during experiment planning. Lighting type must be considered, and maintained from sample to sample, panelist to panelist, and replication to replication. Fluorescent lighting with a color temperature of 3000–3500 K is recommended, including lamps such as Deluxe Warm White, Natural, and Deluxe Cool White. Lamps producing unreal red or pink tints should be avoided. Lighting intensity also is an important consideration and should be maintained at approximately 1614 lux (150 foot candles).

Visual Surface Color Evaluation

Data obtained from hedonic scales are the most appropriate method for predicting consumer satisfaction. However, consumer panels can be costly and often require 50 or more panelists in order to obtain a reasonable indication of purchasing decisions. It is important to note that consumer panels are not recommended for describing meat color, but rather should be used to evaluate acceptance and satisfaction. In contrast to consumer panels, properly trained panelists can describe meat color with a relatively high degree of repeatability and minimal personal bias or preference. However, trained panelists should not be used as indicators of meat product acceptability and consumer preference. A descriptive panel might consist of 4–8 trained panelists, each of which should be screened for color blindness and for their ability to determine color differences using the Farnsworth-Munsell 100-Hue Test (Farnsworth, 1957).

Trained panels can both describe color and quantify discoloration using scales containing descriptors such as red, pink, gray, tan, and brown. Although "discoloration" often refers to a change from desirable oxymyoglobin to undesirable brown metmyoglobin, it is important to note that the term "discoloration" is not always synonymous with visual color transformations associated with myoglobin oxidation. Thus, although scales that estimate percent surface discoloration are available, it is important that panelists understand the definition of discoloration as it pertains to that specific project.

Another obstacle encountered when evaluating meat color is that discoloration often does not occur uniformly over an entire meat surface. For example, browning may occur only in "spots" or localized areas, resulting in a beef steak that has a bright cherry-red surface with a "dime-sized" area of metmyoglobin. In this case, trained panelists can be asked to assign both an "overall" surface color score and "worst point" color score (area at least 1 cm^2 in diameter) to the same steak. Using scales that describe color and quantify surface discoloration, panelists can evaluate color stability during storage or display. In addition, researchers can measure color uniformity or "two-toning" associated with distinct dark and light regions on a steak's surface.

Prior to initiating research projects, it is helpful to conduct orientation sessions that both outline panelist responsibilities and provide "warm-up" samples that represent the numerous colors and various degrees of discoloration that individuals will likely be exposed to during the experiment. In addition, panelists should be asked to evaluate color within the same time frame rather than allowing panelists to assess color at various times throughout the day.

Listed below are examples of visual color scales available to researchers.

Initial color scale:

6 = Very dark purplish-red
5 = Moderately dark purplish-red
4 = Slightly dark purplish-red
3 = Slightly pale
2 = Moderately pale
1 = Very pale

Color uniformity scale:

5 = Extreme two-toning
4 = Moderate two-toning
3 = Small two-toning
2 = Slight two-toning
1 = Uniform color

Descriptive color scale:

8 = Very dark brownish-red or brown
7 = Dark brownish-red
6 = Dark red to reddish-brown
5 = Moderately dark red
4 = Slightly dark red
3 = Dull red
2 = Bright red
1 = Very bright red

Surface discoloration scale:

7 = Total discoloration: 100% of the surface
6 = Extensive discoloration: 80–99% of the surface
5 = Moderate discoloration: 60–79% of the surface
4 = Modest discoloration: 40–59% of the surface
3 = Small discoloration: 20–39% of the surface
2 = Slight discoloration: 1–19% of the surface
1 = No discoloration: 0% of the surface

Consumer preference scale:

8 = Extremely desirable color
7 = Very desirable color
6 = Moderately desirable color
5 = Slightly desirable color
4 = Slightly undesirable color
3 = Moderately undesirable color
2 = Very undesirable color
1 = Extremely undesirable color

Consumer purchasing decisions scale:

5 = Definitely would purchase
4 = Probably would purchase
3 = Borderline purchasing
2 = Probably would not purchase
1 = Definitely would not purchase

Instrumental Surface Color Evaluation

Although visual color evaluations are more closely related to what a consumer may see, the disadvantages associated with conducting visual panels have caused researchers to rely on instrumental measures of color and color stability. Extraction techniques are time-consuming and in most cases, render the sample useless for repeated measures. Thus, due to its rapid and nondestructive nature, reflectance is commonly used to measure surface color. Many researchers note that compared with extraction techniques, surface reflectance more accurately indicates what a consumer may see. The most commonly used reflectance variables are:

L^* = indicates lightness, 100 = white, 0 = black, increased values are lighter;
a^* = measures redness, positive values are red, increased values are more red;
b^* = measures yellowness, increases in b^* are more yellow;
Hue angle = $\tan^{-1} b^*/a^*$, larger angles are more yellow and more discolored;
Saturation index = $(a^2 + b^2)^{1/2}$, larger values represent more intense color.

When utilizing reflectance methodology to characterize surface color, some important considerations include standardization, aperture size, illuminant type, and the angle of observer. For example, when standardizing colorimeters, some researchers recommend covering standardization tiles or glass plates with the same packaging film that the meat samples are packaged in.

The illuminants most commonly used to measure meat color include A, C, and D65. Illuminant A tends to produce a greater proportion of red wavelengths based on color temperature, which is 2854 K compared with approximately 6500 K for Illuminants C and D65. In addition, larger apertures can better detect red color differences.

Most colorimeters and spectrophotometers will measure Hunter *L*, *a*, and *b* values (not designated with *), which are very similar to CIE (Commission Internationale de L'eclairage) values. Given the variety of options available for measuring color, researchers should refrain from comparing color values between different laboratories and projects.

In addition to characterizing surface color, researchers can use reflectance to estimate the amounts of each myoglobin redox form on the surface of meat according to procedures described in AMSA (1991). This methodology allows for a noninvasive, rapid estimation of surface myoglobin redox state using isobestic wavelengths (wavelength at which reflectance is equal for two or more redox forms). Reflectance at isobestic points is converted to *K*/*S* ratios by the Kubelka–Munk equation in order to (1) generate linear data, and (2) account for absorbance and light scattering produced by the muscle's surface and subsurface microstructure (*K* is the absorption coefficient and *S* is the scattering coefficient; Judd and Wyszecki, 1963; Francis and Clydesdale, 1975). These *K*/*S* ratios are subsequently input into the appropriate formulas specified in the Guidelines for Meat Color Evaluation (AMSA, 1991). However, it is important to note that the formulas provided in AMSA (1991) require standard or reference *K*/*S* ratios representing 100% of each myoglobin derivative.

Although reference values are available in the literature, it is recommended that they be derived specifically for each experiment rather than relying on published values. Standards for 100% met-, deoxy-, and oxymyoglobin can be created as follows: To create 100% metmyoglobin, place meat samples in 1.0% potassium ferricyanide for 1 min, drain, blot dry, overwrap in oxygen-permeable film, and incubate at 4°C for 12 h. To create 100% deoxymyoglobin, place freshly cut meat samples in 10% dithionite, drain, blot dry, vacuum package, and incubate for 2 h at room temperature. To create 100% oxymyoglobin, package steaks in 80–100% oxygen and store samples for 2 h at 4°C. Reflectance methodology that differentiates between oxy- and carboxymyoglobin is not available.

Below is a formula that can be used to measure surface oxymyoglobin content. Similar equations are available to estimate deoxy- and metmyoglobin content using $K/S_{474\ nm} \div K/S_{525}$ and $K/S_{572\ nm} \div K/S_{525}$, respectively (AMSA, 1991). $K/S = [(1 - R)^2]/2R$, where R represents the reflectance value.

$$\frac{\text{K/S 610}}{\text{K/S 525}} \text{ for 100\% DMb or MMb} - \frac{\text{K/S 610}}{\text{K/S 525}} \text{ for the sample}$$

$$\frac{\text{K/S 610}}{\text{K/S 525}} \text{ for 100\% DMb or MMb} - \frac{\text{K/S 610}}{\text{K/S 525}} \text{ for 100\% OMb}$$

Determination of Myoglobin Redox State in Meat Extracts

Further details are in Faustman and Phillips (2001).

1. Excise a meat sample that represents the location and pigment of interest (25 g).
2. Homogenize the sample for 30–45 s with 75 mL of 40 mM sodium phosphate buffer (pH 6.8).
3. Filter the homogenate through double-layer cheesecloth.
4. Filter the resulting homogenate (0.40 μm filter).
5. Measure sample absorbance from 500 to 600 nm.
6. Estimate the total concentration of myoglobin using: A525 ÷ (7.6 mM^{-1} × path length in centimeters). Be sure to account for the dilution factor.
7. Calculate the relative proportions of myoglobin redox forms according to Tang et al. (2004).
   - [Deoxymyoglobin] = $[-0.54(A_{582} \div A_{525})] + [1.59 \times (A_{557} \div A_{525})] + [0.55(A_{503} \div A_{525})] - 1.33$;
   - [Oxymyoglobin] = $[0.72(A_{582} \div A_{525})] - [1.43(A_{557} \div A_{525})] - [1.66(A_{503} \div A_{525})] + 2.60$;
   - [Metmyoglobin] = $[-0.16(A_{582} \div A_{525})] - [0.09(A_{557} \div A_{525})] + [1.26(A_{503} \div A_{525})] - 0.52$.

Myoglobin Isolation from Beef Samples

Further details are in Trout and Gutzke (1996).

1. Perform all steps at 4°C.
2. Homogenize 200 g of beef muscle devoid of fat and connective tissue in 600 mL of 10 mM Tris/1 mM EDTA (pH 8.0).
3. Centrifuge homogenate at 3000*g* for 10 min.
4. Adjust supernatant pH to 8.0 with sodium hydroxide and filter through double-layered cheesecloth.

5. Achieve 70% ammonium sulfate saturation of the filtered supernatant, maintain pH, and stir for 1 h.
6. Centrifuge at 18,000*g* for 20 min.
7. Achieve 100% ammonium sulfate saturation of the filtered supernatant, maintain pH, and stir for an additional hour.
8. Centrifuge at 20,000*g* for 1 h.
9. Dialyze precipitated myoglobin (1 volume protein: 10 volume of 10 mM Tris/1 mM EDTA at pH 8.0) for 24 h.
10. Change the buffer every 8 h.
11. Using an appropriate chromatography column and elution buffer, separate myoglobin and hemoglobin, and collect myoglobin-containing fractions.
12. Achieve a desired myoglobin concentration using either a Centricon concentrator or 100% ammonium sulfate saturation.
13. If ammonium sulfate is used, centrifuge at 20,000*g* for 1 h and dialyze as described previously.
14. Purity of the myoglobin extract can be checked by SDS-PAGE and myoglobin solution may be frozen at -80°C for future use.

Preparing Oxymyoglobin in Purified Solutions

The basis for this procedure involves chemical reduction of metmyoglobin with hydrosulfite and then removing unreacted hydrosulfite by chromatography (Brown and Mebine, 1969). To reduce oxidation, care must be taken to keep all solutions in ice.

1. Dissolve myoglobin in an appropriate buffer (citrate for pH 5.6 or sodium phosphate buffer for pH 7.4).
2. Add hydrosulfite (0.1 mg/1 mg myoglobin) to the metmyoglobin solution.
3. Oxygenate deoxymyoglobin by bubbling air through the solution.
4. In order to remove excess hydrosulfite, pass the solution either through a desalting column using gravity as the driving force.
5. Collect the red oxymyoglobin solution as it elutes from the column.
6. Estimate the total concentration of myoglobin using: $A_{525} \div (7.6\ mM^{-1} \times$ path length in centimeters).
7. Calculate the relative proportions of myoglobin redox forms according to Tang et al. (2004).

Preparing Carboxymyoglobin in Purified Solutions

Further details are in Suman et al. (2006b).

1. Obtain deoxymyoglobin via hydrosulfite-mediated reduction (0.1 mg sodium hydrosulfite to 1 mg myoglobin).
2. Bubble deoxymyoglobin with carbon monoxide and monitor the conversion of deoxymyoglobin to carboxymyoglobin spectrophotometrically. Typical bubbling time with 0.4% CO is 40 min.
3. For 100% carboxymyoglobin, the magnitudes of absorbance at 543 nm will be greater than at 581 nm. Thus, the ratio of A_{543}/A_{581} for a solution of 100% carboxymyoglobin will be greater than 1, whereas the ratio will be less than 1 for 100% oxymyoglobin.

4. Remove residual hydrosulfite by passing the carboxymyoglobin preparation through a PD-10 column. Be sure to use buffer that was bubbled with CO in order to minimize the conversion of carboxymyoglobin to oxymyoglobin.
5. Following PD-10 treatment, desalted carboxymyoglobin samples should be briefly re-bubbled with CO for an additional 10 min.
6. Although methodology is not available to quantify myoglobin redox state in solutions containing carboxymyoglobin, pigment oxidation can be indirectly estimated using the ratio of A_{503}/A_{581} (a greater value indicates more oxidation).

Metmyoglobin Reducing Activity (MRA)

Further information is in Sammel et al. (2002).

1. Remove a 2.54 × 2.54 × 1.27 cm meat sample.
2. Submerge samples in a 0.3% sodium nitrite solution for 20 min to form metmyoglobin.
3. Remove samples from the nitrite solution, blot dry, and vacuum package.
4. Measure prereduction metmyoglobin content in vacuum-packaged samples.
5. Incubate vacuum-packaged samples at 30°C for 2 h to induce metmyoglobin reduction.
6. Measure postreduction metmyoglobin content on vacuum-packaged samples.
7. Calculate metmyoglobin reduction activity as:
   - absolute MRA = prereduction MMb − postreduction MMb;
   - relative MRA = (prereduction MMb − postreduction MMb) ÷ prereduction MMb.
   - Alternatively, resistance to nitrite-induced MMb formation as measured by prereduction MMb can be used to estimate MRA.

Alternatively, researchers also can measure MRA according to McKenna et al. (2005).

1. Homogenize 5 g of finely minced muscle with 25 mL of 2 mM phosphate buffer (pH 7.0).
2. Centrifuge the homogenate at 35,000*g* for 30 min.
3. Filter the supernatant using Whatman #541 filter paper.
4. Add 1–2 crystals of $K_3Fe(CN)_6$ to the filtered supernatant and dialyze against 2 mM phosphate buffer (pH 7.0).
5. Combine 0.2 mL of the dialyzed muscle extract with 0.1 mL of 5 mM disodium EDTA, 0.1 mL of 50 mM citrate buffer (pH 5.6), 0.1 mL of 3 mM potassium ferricyanide, 0.3 mL of 0.1 mM bovine metmyoglobin, and 0.1 mL distilled-deionized water.
6. Initiate the reaction with 0.1 mL of 10 mM NADH and measure absorbance at 580 nm (30°C).
7. Calculate MRA during the linear phase of the reaction.

Aerobic Reducing Activity

Further details are in McKenna et al. (2005) and Ledward (1972).

1. Remove a 2.54 × 2.54 × 1.27 cm meat sample and overwrap in oxygen-permeable film.
2. Incubate samples in 1% oxygen (remainder nitrogen) for 24 h to induce metmyoglobin formation.

3. Remove the sample from the 1% oxygen environment and measure surface metmyoglobin using reflectance.
4. Incubate samples for an additional 24 h in the atmosphere.
5. Measure surface metmyoglobin and calculate aerobic reducing activity using the decrease in metmyoglobin during atmosphere exposure.

Oxygen Consumption

Further details are located in Madhavi and Carpenter (1993).

1. Oxygenate a deoxygenated meat sample at 1°C for 30 min in the atmosphere.
2. Measure oxymyoglobin on the oxygenated surface using reflectance.
3. Vacuum package the sample and incubate at 4°C.
4. At 10 min intervals for 1 h, measure the loss of oxymyoglobin on the surface of each vacuum-packaged sample.
5. Oxygen consumption can be indirectly determined using the accumulation of deoxymyoglobin during incubation at 4°C.

Alternative methods are in McKenna et al. (2005) and Sammel et al. (2002).

1. Place a muscle sample (not previously exposed to air) in a vacuum barrier pouch.
2. Flush and fill the pouch with 1% oxygen (remainder nitrogen) and seal.
3. Using a headspace analyzer, measure the oxygen and carbon dioxide concentrations in the pouch. Be sure to use an adhesive tape that allows multiple measurements.
4. Incubate samples at 35°C for 2 h.
5. Following incubation, measure the oxygen and carbon dioxide concentrations in the pouch.
6. Changes in package headspace gas composition can be used to measure oxygen consumption.
7. An alternative method has been proposed using polypropylene bottles with modified caps, rather than vacuum barrier pouches.

Surface Myoglobin Oxygenation

Further details are located in Brewer et al. (2001).

1. Determine surface myoglobin redox status immediately after cutting or grinding a meat product. Be sure that the initial color measurement occurs immediately after cutting in order to minimize the exposure of deoxygenated pigments to the atmosphere.
2. Every 5 min for the first 0.5 h after cutting or grinding, measure the percentage of each myoglobin form on the freshly cut surface.
3. Changes in $L^*a^*b^*$ can also be used.

Depth of Oxygenation

Further details are located in Ramanathan et al. (2009).

1. Following an appropriate amount of oxygen exposure (timing is dependent on project objectives and design), cut meat samples perpendicular to the oxygenated surface.

2. After samples are cut in half, the depth of oxygen penetration can be measured immediately using calipers.
3. Depth of oxymyoglobin (in millimeters) is inversely related to oxygen consumption.
4. A similar approach has been used to measure the depth of carbon monoxide penetration in steaks and ground beef (Jayasingh et al., 2001).

Alternatively, researchers can use procedures described in McKenna et al. (2005).

1. Obtain thin slices of freshly cut, deoxygenated muscle (approximately 1 cm thick).
2. Prior to oxygenation, immediately place the slices between two glass plates.
3. Cover the glass with oxygen-permeable overwrap and store at 4°C.
4. Determine depth of oxygen penetration by measuring the thickness of the oxymyoglobin layer using digital calipers.

Mitochondrial Isolation and Oxygen Consumption Measurement

Further details are located in Smith (1967). Be sure to use ice to limit temperature fluctuation during mitochondrial isolation.

1. Wash ground cardiac muscle (100 g) twice with 250 mM sucrose.
2. Add 200 mL of mitochondria isolation buffer (250 mM sucrose, 10 mM HEPES, 1 mM EGTA, and 0.1% BSA, pH 7.2).
3. Add nagarase protease (protease/tissue, 0.5 mg/g) and stir slowly for 20 min. Maintain pH between 7.0 and 7.2.
4. Dilute the suspension with 1000 mL of isolation buffer.
5. Homogenize using a Kontes Duall grinder, followed by a Wheaton Potter-Elvehjem grinder, three passes each.
6. Centrifuge the homogenate at 1200*g* for 20 min.
7. Filter the supernatant through cheesecloth to remove visible fat.
8. Centrifuge the resulting supernatant at 26,000*g* for 15 min.
9. Wash the pellet twice with mitochondria suspension buffer (250 mM sucrose, 10 mM HEPES, pH 7.2).
10. Determine mitochondrial protein content using a Bicinchoninic Acid Protein Assay Kit.
11. Mitochondrial oxygen uptake can then be measured using a Clark oxygen electrode.
12. Add mitochondria to the incubation chamber and maintain the temperature at 25°C.
13. Record oxygen consumption over time, maintaining the pH at 7.2.
14. States III and IV oxygen consumption rates (OCR) and respiratory control ratio (RCR) can be calculated:
    - State IV data characterize the OCR of isolated mitochondria in the presence of added substrate;
    - State III data characterize the OCR of isolated mitochondria in the presence of both substrate and ADP;
    - The RCR determines the functional integrity of isolated mitochondria and is calculated as state III ÷ state IV.

Cooked Color

Denatured myoglobin is responsible for the appearance of cooked meat products. In general, consumers typically associate a brown color in the center of beef products with temperatures sufficient to inactivate food-borne pathogens. However, the effects of various factors on raw meat pigment chemistry can translate into cooked color concerns such as premature browning.

Premature browning is a condition in cooked beef where myoglobin denaturation occurs at a temperature lower than that necessary to inactivate pathogens. As a result, the relative brown color of cooked meat may not be a reliable indicator of product pasteurization. Several intrinsic (myoglobin redox state, muscle source, and antioxidants) and extrinsic (packaging, storage, and cooking from a frozen state) factors influence the susceptibility of beef to premature browning.

One factor influencing the incidence of premature browning is the redox state of myoglobin in raw beef prior to cooking (Hague et al., 1994). More specifically, raw beef containing metmyoglobin will prematurely brown when compared with raw beef containing predominantly ferrous myoglobin (Lavelle et al., 1995; Warren et al., 1996). This occurs because the temperature at which myoglobin denatures is dependent on the protein's redox status, with the relative resistance of myoglobin to heat-induced denaturation as follows: deoxy > oxy > metmyoglobin (Machlik, 1965). The practical implication of this is that vacuum packaging will decrease the occurrence of premature browning compared with aerobic packaging. Furthermore, high-oxygen packaging increases premature browning compared with PVC overwrap (Seyfert et al., 2004a, 2004b; Suman et al., 2005). In contrast, packaging containing CO will minimize the occurrence of premature browning (John et al., 2004, 2005).

Similar to raw color evaluation, trained panelists can use color descriptors such as red, pink, tan, brown, gray, or white to describe the doneness of cooked meat.

Internal cooked beef color (Seyfert et al., 2004a):

6 = Dry, brown throughout (well done)
5 = Tan/brown center and edges, no evidence of pink
4 = Slightly pink center, light brown to tan edge (medium)
3 = Pinkish-red center, pink to light brown/tan to outer surface
2 = Reddish-pink center, pink border, tan edge
1 = Raw red center, pink border, tan edge (medium rare)

Internal cooked pork color (Lien et al., 2002):

5 = Tannish white, no evidence of pink
4 = Very slight pink to tannish white
3 = Slightly pink
2 = Moderately pink
1 = Purplish-red to pinkish-red

In addition to visual assessment of cooked color, researchers can use instrumental variables such as reflectance. For example, a^* and hue angle can be used to evaluate premature browning. Cooked color also can be evaluated using the difference in reflectance at 630 nm and 580 nm, with larger values indicating a more red color.

Percentage thermal pigment denaturation also can be used in cooked color experiments and is commonly expressed as the ratio of extractable myoglobin in cooked samples to extractable

myoglobin in raw samples. The following procedure can be used to assess myoglobin denaturation as described by Hunt et al. (1999) and Warriss (1979):

1. Pulverize 8 g of sample.
2. Add 40 mL of 40 mM potassium phosphate buffer (pH 6.8).
3. Blend for 1 min.
4. Store in the dark for 1 h at 4°C.
5. Centrifuge at 15,000*g* for 30 min.
6. Filter the resulting supernatant.
7. Add a 10% sodium hydrosulfite solution to the supernatant.
8. Measure absorbance at 433 nm.
9. Calculate myoglobin concentration using absorbance at 433 nm, 16,800 for the molecular weight of myoglobin, 114×10^3 cm/mM as the extinction coefficient, and the appropriate dilution factor.
10. Calculate percent denatured myoglobin using the following: [(myoglobin recovered from uncooked patties − myoglobin recovered from cooked patties) ÷ myoglobin recovered from uncooked patties] × 100.

Iridescence

Iridescence is a physical phenomenon characterized by a shiny, rainbow-like appearance on the surface of cooked meat products that is often misinterpreted by consumers as an indication of chemical additives and/or microbial spoilage. Several colors result in a kaleidoscope-like appearance, including green, red, orange, and yellow. It is important to note that myoglobin likely plays a negligible role in iridescence. In contrast, the mechanism of iridescence is primarily associated with product surface microstructure, light diffraction, and muscle fiber orientation (Swatland, 1984; Wang, 1991; Lawrence et al., 2002; Kukowski et al., 2004). As a result, structural uniformity on the surface of a muscle food will produce light diffraction patterns associated with iridescence whereas disruption of surface microstructure limits iridescence (Wang, 1991). Researchers have used trained panels to quantify the amount, intensity, and surface area coverage associated with iridescence (Lawrence et al., 2002).

Iridescence intensity:

5 = Very strong iridescence
4 = Strong iridescence
3 = Moderate iridescence
2 = Very slight iridescence
1 = No iridescence

Surface area covered by iridescence:

6 = 81–100% of surface area
5 = 61–80% of surface area
4 = 41–60% of surface area
3 = 21–40% of surface area
2 = 1–20% of surface area
1 = No iridescence

Cured Color and NO-Myoglobin

Nitrite is added to cured meat products in order to (1) inhibit the outgrowth of *Clostridium botulinum* spores, (2) fix the characteristic pink color associated with cured meat, and (3) serve as an antioxidant. It is important to note that nitrite does not react with myoglobin whereas nitric oxide can bind to myoglobin and form ferrous NO-myoglobin. Subsequent heating/cooking denatures NO-myoglobin, exposing the centrally located pink porphyrin ring. However, cured meat color is susceptible to light and oxygen and will fade to gray if not vacuum packaged. Both intensity and fade of cured color can be determined using the ratio of reflectance at 650 nm and 570 nm where a numerical decrease represents cured color fade. Researchers also can visually evaluate cured color intensity and cured color fade using the following:

6 = Very intense cured color
5 = Intense cured color
4 = Small cured color
3 = Slight cured color
2 = Very slight cured color
1 = No cured color

In addition to visual evaluation of cooked color, percent nitrosohemochrome can be measured, according to Hornsey (1956). It is important to note that light exposure during sample preparation and incubation should be minimized to limit color fade. NO-heme concentration can be determined according to the following.

1. Combine 2 g of sample with 9 mL of 90% aqueous acetone.
2. Mince samples and place in a glass test tube. Make sure tube caps are sealed.
3. Incubate samples in the dark at 25°C for 10 min.
4. Filter samples using Whatman no. 42 filter paper.
5. Measure absorbance at 540 nm using aqueous acetone as the blank. Avoid disposable cuvettes to minimize turbidity.
6. Calculate the concentration of NO-heme as $A_{540 \times 290}$ ppm.

Bone Marrow Discoloration

Disruption of red blood cells during product fabrication releases hemoglobin, which results in marrow darkening via methemoglobin oxidation (Gill, 1996). Research suggests that the aqueous phase of bone marrow is the principal determinant of marrow color (Mancini et al., 2004, 2005). Thus, maintenance of ferrous hemoglobin on the surface of cut bones plays a critical role in improving the shelf life of bone-in meat products. As a result, water-soluble reducing agents such as ascorbic acid and sodium erythorbate or modified atmosphere packaging containing 0.4% carbon monoxide can lengthen marrow color stability. Conversely, high-oxygen packaging tends to promote bone marrow discoloration (Mancini et al., 2005; Grobbel et al., 2006; Raines et al., 2006).

Evaluation of marrow color can be accomplished using visual and instrumental techniques described in Mancini et al. (2004) and Grobbel et al. (2006). The following visual color scales can be used to assess bone marrow packaged in high oxygen:

Vertebrae marrow color:

7 = Black discoloration
6 = All gray or grayish-black
5 = Moderately gray
4 = Grayish-pink or grayish-red
3 = Slightly grayish-pink or grayish-red
2 = Dull pinkish-red
1 = Bright reddish-pink to red (typical freshly cut bone)

Amount of bone surface discoloration:

Discoloration can be considered atypical freshly cut marrow color such as gray, black, brown, or tan.

7 = Total surface discoloration
6 = 80–99% discoloration
5 = 60–79% discoloration
4 = 40–59% discoloration
3 = 20–39% discoloration
2 = 1–19% discoloration
1 = No surface discoloration

An extraction technique for bone marrow is located in Grobbel et al. (2006) and Calhoun et al. (1998).

1. Expose marrow within lumbar vertebrae bone pieces by cutting samples in half.
2. Place samples on a screen located within a centrifuge bottle.
3. Centrifuge samples at 8075*g* for 25 min.
4. Filter extracted marrow through a nylon-mesh (% open area 47).

New Developments in Color Research Using Proteomics

A recent trend in meat color research has involved the use of proteomic techniques to better understand the interrelation between lipid and pigment oxidation. This research suggests that secondary lipid oxidation products accelerate heme protein oxidation via covalent modification of amino acids (Faustman et al., 1999). In particular, 4-hydroxyn-nonenal adduction has been confirmed by tandem mass spectrometry and proteomic data-mining to occur at nucleophilic histidine residues (HIS 24, 64, 93, 116, 119, and 152) via Michael addition (Alderton et al., 2003; Lee et al., 2003; Suman et al., 2006a, 2006b).

Conclusion

Meat color is not determined solely by one factor, but by several factors that operate in concert. Nevertheless, knowledge of both ante- and postmortem factors that affect color is necessary to improve product shelf life. A solid foundation in myoglobin chemistry will benefit research focused on meat color. However, there are several techniques available for measuring color stability, and

determining the ideal procedure is often not straightforward and depends on several factors, including the experimental design and project objectives.

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10 Meat Cookery

Clinton Rowe and Chris R. Kerth

Meat cookery is generally thought of by the non-meat scientist as a simple way to prepare meat to add flavor, texture, and eliminate potential harmful pathogens. However, meat cookery is very complex with regard to the type and the expected outcomes. As most people know, there are many different ways to cook meat products, and the different cookery methods impart differing characteristics to the meat. Grilling, for example, uses dry high heat to cook, whereas a Crockpot uses moist low heat. The characteristics are vast and differing muscles are better suited for the different types of cooking. This chapter will include the differing types of cookery as well as the types of meat to use with each.

Muscles within the body are composed of different constituents, as covered in Chapters 2 and 3. Muscles used for locomotion generally have higher amounts of connective tissue than that of the muscles that are used for support. Moreover, these muscles used for locomotion have differing amounts of fiber type distributions, which affect the amount of connective tissue and lipid content of the muscle. This all has impacts on the palatability of the muscles and that is why different cookery methods are needed.

Cooking Loss

Cooking loss is a major issue that is associated with degree of doneness, especially in steaks. The reasoning it is more associated with steaks is due to the fact that ground meat, chopped and formed products, and non-intact products are to be cooked to a minimum of 160°F. Therefore, cooking loss is generally of greater interest in whole muscle cuts. Dry cookery methods increase the amount of cooking loss due to the gradient in humidity. As with almost all things in nature, humidity tries to stay at equilibrium. Therefore, when the meat product is heated to temperatures capable of evaporating fluids, the liquids leave the muscle creating moisture loss. However, this is not the only reason for cooking loss. The main reason is that of steric effects in the muscle. When the muscle is cooked, it causes the proteins in the muscle to change in shape and conformation. This change in conformation causes them to shift and lose the ability to bind and hold onto water. This loss of ability allows for the cooking loss to occur.

Cooking loss is affected by many factors. These factors include final cooking temperature, pH of the muscle, and method of cooking. Higher final cooking temperatures increase the amount of water that is driven from the muscle as well as the amount of fat or lipid that is liquefied and allowed to

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excrete. Degree of doneness or final endpoint temperature has a direct effect on cooking loss. As the final endpoint temperature increases, the amount of cooking loss increases as well, as shown by Obuz et al. (2004) using the *longissimus lumborum*, *deep pectoralis*, and the *biceps femoris.* The pH of the muscle also has a large role in the amount of cooking loss of a muscle. The pH is directly related to that amount of negative charges on the muscle proteins that bind the water molecules. When pH is close or at the isoelectric point (pI), the point in which in the net charge of the protein is equal, less water is allowed to bind. This occurs due to the lack of repulsion of the muscle fibers that allows space for the water to be trapped in the muscle matrix and also due to the lack of available charges to bind and hold on to water. Likewise, muscles that have an extreme high pH (those above 6.0) have the ability to retain a larger amount of water. In beef, it has been found that as pH increases, cooking loss decreases in roasts from the *semitendenosus* and *longissimus* muscles (Hawrysh et al., 1985). Therefore, muscle pH that is above the pI has the ability to bind water. It must be noted however, that the pI of meat is ∼4.5, and meat has a large buffering capacity and moving the pH that low is tough, even in the worst of conditions. Minor changes in pH can affect cooking loss and ultimately juiciness of the meat.

Cooking method also has a large role in the amount of cooking loss. Depending on the method, cooking losses can be large or small. Differences in cooking methods will be covered more thoroughly in the Cookery Methods section that follows.

Maillard Reaction and Flavor Impacts

The flavor of cooked meat is the flavor that has drawn people to cook steaks, chicken, and hamburgers for years. This flavor is not a given for these products and can be changed drastically depending on the type of method used. There are differing ways to impart flavor on meat products when cooked. The Maillard reaction is the first of which will be discussed.

The Maillard reaction occurs when there is an interaction between an amine group and a reducing sugar that go through three main steps to give color, flavor, and aromatics or odor characteristics. The reaction as described in the previous sentence is extremely oversimplified, but the exact chemistry of the reaction is not necessary for the understanding of the end products. This reaction produces colored products that are high in molecular weight, generically called melanoidins, and are responsible for the browning mechanism in meats (Jousse et al., 2002). It also produces the flavor and aromatic products that are associated with meat flavor (van Boekel, 2006).

The Maillard reaction is the reaction that occurs when meat is subjected to high cooking temperatures. The Maillard reaction occurs at ∼310°F. Since water boils at 212°F, meat products that are cooked in water do not exhibit the products that are normally associated with that of the Maillard reaction.

Other ways of imparting flavor to meat is through the use of smokes or aromatics and other flavor enhancers used in the cooking process. As most know, the flavor profile that a charcoal grill imparts is quite different from that of a propane grill or electric grill. These flavor profile constituents have to do with the differences of what particulates are in the smoke. Charcoal grills impart a woody or smoky flavor to the products that are cooked on them and most people or consumers find the flavor pleasing. The type of flavor from these charcoal grills can be changed depending on the type of charcoal used, as well as by the heat of the charcoal. The heat of the charcoal has an impact due to the amount of exposure time that the meat has to the heat source. When the charcoal is burned in a grill, the production of aromatic compounds occurs. These aromatic compounds bind with the proteins in the meat to produce the pleasing flavor that consumers desire. Propane or gas grills can

also impart flavor, but most do not impact the eating experience due to the low relative amount of aromatic compounds produced. Smoking meat or the use of smoke houses can also impart flavor to the meat and will be discussed more thoroughly in the Smoking section of this chapter.

Warmed over flavor is a common flavor among reheated products. When meat is cooked, the proteins are denatured. This denaturation of proteins allows for inorganic chemicals such as iron to be released. Iron is a prooxidant, meaning that it has the affinity to take electrons from other molecules. These molecules such as fatty acids, myoglobin, and sarcoplasmic and myofibrillar proteins can be oxidized readily. The release of electrons, especially in fatty acids cause rancidity and can give the meat a pungent odor and taste. Most heat and eat entrees have some sort of antioxidant to halt the process. Oxidation in cooked meat can be 10 times that of fresh meat.

Soluble and Insoluble Collagen

Over the years, it has been recognized that not all cuts of meat are suitable for steaks. As discussed in Chapter 6, collagen or connective tissue differs in content and structure depending on muscle location and type. The various cuts or primals especially in beef can exhibit different characteristics. These various characteristics differ due to the location of the muscle and also due to activity. The middle meats, or the *longissimus dorsi* and *psoas major* contain the ribeye, strip loin, filet, and T-bone. These cuts are very tender with low amounts of connective tissue and make very good grilling steaks. However, muscles from the round generally have high amounts of connective tissue and grilling causes them to be extremely tough. Muscles that are high in connective tissue have traditionally either been put into ground products or roasts. The reasoning behind using these products for roasts is that the slow, moist low heat helps in solubilizing the connective tissue in the muscle.

Collagen represents approximately 2% of the protein in muscle, but has a large impact on the texture changes that occur during heating (Powell et al., 2000). Collagen goes through structural denaturation and solubilization during heating; and the rate and extent of these changes depend on many factors including maturity of the collagen, heating rate, relative humidity, and restraint during cooking (Powell et al., 2000). When temperatures are between 53°C and 63°C, collagen denaturation occurs, which most likely first involves the breaking of hydrogen bonds that allow loosening of the fibrillar structure followed by the contraction of the collagen molecule (for review, see Tornberg, 2005). If the collagen is unrestrained, the fibers shrink to one-quarter of its resting length when heated to temperatures between 60°C and 70°C (for review, see Tornberg, 2005). If the collagen fibers are not stabilized by heat-resistant intermolecular bonds, they dissolve and form a gelatin upon further heating (for review see Tornberg, 2005).

This occurs because the high humidity within the cooking environment as well as the heat swells the collagen and allows the collagen to start to dissociate. This dissociation of the collagen tenderizes the meat and makes the meat product more palatable. The process is slow and, depending on the muscle and species used, can take 12 h or more. More importantly, for cooking these meats, time, temperature, and humidity is critical.

Collagen molecules are extremely strong and hard to break down. As the animal ages, the amount of soluble bonds decreases, and the amount of insoluble bonds increases. There are several factors that cause the shift in the amount of bonds. Feeding level and intensity can have a large impact as well as age of the animal. It has been shown in research that on feeding older cattle high-energy diets, these permanent crosslinks in the collagen can be replaced with soluble bonds. As the feeding levels increase, the amount of energy in the diet increases. This increase in diet speeds up the natural process of protein turnover. The older insoluble bonds are broken down by the natural proteases in

the animal and new bonds replace them. However, if the bonds are not replaced and a large amount of collagen and insoluble bonds exist, no amount of aging time is sufficient to increase tenderness past a certain undetermined point. The proteases involved in the breakdown of proteins postmortem do not breakdown collagen. Therefore, on cuts that are high in connective tissue, both soluble and insoluble, aging has a minimal effect.

Cookery Methods

Fast versus Slow Cookery

Many different types of cookery methods exist and the best one to use largely depends on the muscle cut. However, the question always arises on whether to cook a piece of meat fast or slow. It is generally recognized that moist heat cookery is generally slow cooked and dry heat cookery is fast, but this is not always the case. The reasoning is due to several different factors including type of heat transfer and ambient humidity. Moist heat cookery can be used to cook meat very fast and dry heat cookery can be used for a slow-type cook.

Use of either heat can yield differing results. Research has shown that using roasts from the rib and round of beef, tenderness profiles can be changed. It has been found that the muscle cuts subjected to the slower cooking regimen produced more tender meat (Bayne et al., 1969). In this study, they used cooking temperatures, 93°C and 149°C; and the cuts from the round took ∼2.5 times longer to cook and the cuts from the rib took ∼1.5–1.75 times longer when cooked at the lower temperature than the cuts at the higher temperature (Bayne et al., 1969). In that study, they concluded that the added tenderness was due to the degradation or solubilization of collagen. As discussed previously, the solubilization of collagen is increased by a slower cooking rate.

Dry Heat Cookery

Dry cookery methods are methods that use dry high heat. Dry cookery methods include grilling, roasting, broiling, and baking. Grilling utilizes high heat that sears the outside of the meat, thereby locking in the juices within the muscle. This type of cooking generally cooks the meat quickly and can impart flavor through the Maillard reaction discussed in the Maillard Reaction and Flavor Impacts section. Utilizing this cookery method, however, does not solubilize collagen which will be covered in the Moist Heat Cookery section. Cuts of meat that are ideal for these types of cookery are steaks that are low in connective tissue from muscles such as the *psoas major, longissimus dorsi*, and the *infraspirnatus*. Also, hamburgers and ground product also benefit from high heat from searing the outside. Additionally, the protein in the muscles coagulate to help hold the product together as is the case with hamburgers, sausages, and chopped and formed products and products stuffed into casings.

The heat from cooking causes a structural change to the proteins in the muscle. When exposed, the bonds within the protein are broken and new bonds are formed. For products that are comminuted or reformed such as boneless hams, deli loafs, cased sausages, and other products that need to stay together, this process aids in cohesion. In brief, the proteins are extracted from the interior and surface of the muscle. The close proximity and pressure of the casing or formed product with heat allow for new bonds to be formed. This cohesion of the proteins is what gives and is referred to as mouth feel.

Moist Heat Cookery

Moist heat cookery uses either high humidity or liquid to cook the meat. Examples of moist heat cookery are smokehouses set to have high humidity, stewing, deep frying, boiling, and pan frying in a liquid. Moist heat cookery has several advantages and impacts on meat texture and flavor. The liquid that is used to cook with can add flavor or color to the product. However, when cooking with some liquids, mainly that of water, the Maillard reaction will not occur. This is due to the fact that the boiling point of water is much lower than the temperature needed to achieve the reaction. Oils, on the other hand, have a much higher boiling point. Therefore, use of deep fryers or oils as the cooking liquid, the Maillard reaction can be achieved. This helps to add both flavor and texture to a product.

Moist heat cookery does have benefits over that of dry heat cookery. The benefit is mainly the breakdown of collagen or connective tissue. As discussed in the Soluble and Insoluble Collagen section, moist heat has the ability to swell the collagen and break it down so that the meat is more tender. It also has an effect on the texture of the meat. The breakdown of the connective tissue in the muscle causes the meat to lose its structural integrity. This is the reasoning that roasts cooked in a crock pot, or meat cooked in a smoke house for hours, has a completely different texture and appearance. Pulled pork or beef is an example of the loss of structural integrity; the connective tissue has been solubilized and the remaining structure is not able to hold the product together, so the meat more or less falls off of the bone.

Smoking

Smoking is perhaps the oldest form of meat preservation. Many years ago, people discovered meat that was smoked lasted longer than that of fresh meat. The chemical compounds of the smoke bind with the proteins to form a stable product. Moreover, the low heat helps to dehydrate the meat. Combined with the smoke and low water activity, bacteria and other pathogens are not able to grow. This preservation effect of smoke is still used today.

Smoking involves several different forms or types of cookery. Smokehouses of today are designed to control the humidity of the environment so that both dry and moist heats can be applied. Smokehouses generally start with a dry heat so that the surface of the product is dried when the application of smoke occurs. This aids in the binding properties of the smoke with the proteins in the meat. After the smoke cycle, moist heat is then applied to cook the product more thoroughly.

Smokehouses are, for all intents and purposes, large convection ovens. Cooking speed can be controlled by the amount of humidity in the smokehouse. Cooking can be done faster with a moist heat than with a dry heat. This has to do with evaporation of liquids. It can be compared with sweating. It is easier to cool down in a dry environment when sweating because as the water or sweat evaporates, heat is expelled. This dispersion of moisture with heat helps cool. Likewise, sweating in a humid environment does little in the way of cooling. This is because the ambient air is already saturated with moisture making it harder for sweat or moisture to evaporate. The lack of evaporation does not allow for the dispersion of heat and therefore the heat remains. Same is true with meat products. Since meat is approximately 70–75% water, moisture loss comes readily. When fluids from the meat travel from the inside of the meat to the outside, they are allowed to evaporate if the environment is dry. This has a cooling effect on the meat and slows cooking time because heat is lost. Likewise, when the migration of fluids occurs in a moist air environment, the fluids cannot

evaporate, thereby retaining the heat in the product and decreasing cooking time. Additionally, the amount of cooking loss can be mitigated with the application of moist heat.

The generation of smoke is done by burning wood. Differing woods have different aromatic compounds and therefore can have different flavor profiles in meat. There are two main types of smoke: liquid or natural. Regardless of the type of smoke, wood must be burned. Liquid smoke is produced by burning wood and capturing it using water as a filter. The excess water is then removed from the smoke and a condensed form of smoke is made in the liquid. Using an atomizer, small smoke particles can be made. These particles then stick to the surface of the meat. The end result is the smoke flavor and color that consumers have become accustomed too.

Natural smoke is smoke that is generated by burning wood and is directly pumped into the smokehouse. The fine particulates in the smoke bind to the proteins and fat in the muscle. This binding of the smoke changes the appearance and flavor of the meat.

Microwave

Radiation, or microwave, cooking is utilized on differing products. Most meat products can be cooked using radiation, but the texture and flavor of the meat can be different. One example of meats that are microwaved is ready-to-eat bacon found in grocery stores. At the plant, the bacon is sliced, put onto a conveyor and sent through the microwaving process. The radiation waves cook the bacon, it is then packaged and sent out to consumers to use on any product they desire.

Microwaves cook by means of electromagnetic energy in the frequency range of 300–3000 MHz, and can be used successfully to heat many dielectric materials (Bradshaw et al., 1998). Domestic microwaves operate at 2450 MHz with the main heating mechanism for dielectric heating being dipolar loss, or the re-orientation loss mechanism (Bradshaw et al., 1998). When the material, or food containing permanent dipoles is subjected to a varying electromagnetic field, the dipoles are unable to follow the rapid reversals in the field (Bradshaw et al., 1998). Due to the phase lag, power is dissipated in the material causing heat (Bradshaw et al., 1998).

Cooked Color

Cooked color is different from that of fresh meat color. When meat is cooked, myoglobin, which is a protein, is denatured and experiences a structural change. The loss of structural integrity in the porphrin ring allows the heme portion to become exposed and release the oxygen and turn a brown color. Cooked color has also been used for years to determine doneness. However, this is not always the case. High-pH meat such as dark cutters or DFD (dark, firm, and dry) in beef has the ability to retain bound oxygen on the myoglobin and retain a pinkish-red color when cooked. Likewise, different conditions will cause premature browning in meat. This premature browning can cause great problems, especially in ground or non-intact product due to the possibility of adulteration or pathogens. The lower cooking temperature would not be sufficient to destroy the pathogens and would allow for contamination.

It has been found that the oxymyoglobin and deoxymyoglobin are more prone to denaturation than that of deoxymyoglobin, regardless of pH (Hunt et al., 1999). The susceptibility of myoglobin to denaturation and color change are dependent on pH, ligand bound to the heme iron, and the stability of the globin chain (Hunt et al., 1999).

Conclusion

Meat cookery is very complex and the type needed is dependent on the type of results desired. The differing types of cookery allow for the optimal eating experience to the consumer. Likewise, utilizing the wrong cookery method with a piece of meat can render it unpalatable and unacceptable. It is of great importance with the amount of varying cuts of meat to recognize the difference and utilize it to the highest degree.

Cooking can add great value to a cut, but more importantly it renders it safe for human consumption by destroying potential pathogenic bacteria. It is important to realize the nature of the muscle depends on the antemortem effects and that not all meat is the same. With that said, most meat is fairly uniform, but differences in pH and other factors can make large differences in texture, juiciness, and overall palatability. It should also be remembered that cooked color is not always a good indicator of doneness, both for establishing whether it is safe to consume or for steaks the desired level of doneness. Meat is one of the most nutritious and best tasting foods known to man. It is this knowledge that allows us to find enjoyment in the simple pleasures of grilling a steak or browning ground beef.

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11 Trained Sensory Panels

Christy L. Bratcher

Introduction and History

There have been many articles and books published to discuss the history, design, and follow through of sensory panels (Peryam, 1990; Stone and Sidel, 1993; Lawless and Heymann, 1999; Meilgaard et al., 2007). This is just a brief list of references that contain the masses of information and within each of these references there are countless contributions of those from whom that information was compiled. The evolution of formal sensory evaluation began in the 1930s and 1940s and has been growing ever since. Sensory evaluation has become so important from a research aspect that there are M.S. and Ph.D. programs which specialize in training adept sensory analysts. In addition to specialized training, there has been a development of journals specific to sensory science which has greatly impacted the field, as well as symposiums, conferences, and specialized sections of larger organizations such as the Institute of Food Technologists. In an attempt to create a concise application of sensory analysis as it pertains to meat science, the "authorities" on sensory evaluation will be paraphrased in the following text.

Sensory evaluation comprises a set of techniques for accurate measurement of human responses to foods and minimizes potentially biasing effects (Lawless and Heymann, 1999). A definition that has been adopted by many is that sensory evaluation is a scientific discipline used to evoke, measure, analyze, and interpret reactions to those characteristics of food and materials as they are perceived through the senses of sight, smell, taste, touch, and hearing (Anonymous, 1975). This definition encompasses all of the senses, not just taste (Stone and Sidel, 1993).

Because instrumental assessments give values that miss the important perceptual process of the interpretation of sensory experience by the human brain before responding (Lawless and Heymann, 1999), it is important to collect sensory data to make value of the human response. Sensory tests are conducted to study how product manipulations will create perceived changes to human observers (Lawless and Heymann, 1999). Sensory testing goes as far back as there were humans to evaluate the goodness or badness or anything that could be consumed (Meilgaard et al., 2007). Sensory testing can establish the worth or acceptability of a commodity as was seen by the rise of the trading industry (Meilgaard et al., 2007). Buyers would test a small sample of a load and sellers would begin to set their prices on the basis of an assessment of the quality of the goods (Meilgaard et al., 2007).

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Trained Panels and Sensory Attributes

There are three classes of test methods in sensory evaluation: (1) discrimination, (2) descriptive, and (3) affective. Affective tests are generally indicative of a consumer panel and indicate preference or acceptance of a product. Discrimination tests are utilized to determine if products are different from one another, and descriptive tests describe sensory properties and their perceived intensities. For trained panels, the focus will be mostly on descriptive; however, there are some applications where discrimination is appropriate. Discrimination testing using techniques such as triangle tests or sorting tests can also be used during training of descriptive panels.

The difficulty in sensory testing arises from pitfalls in the variability of humans as test instruments. Any sensory test will be affected depending on the extrinsic and intrinsic factors that surround a particular test instrument (panelist) on any given day. For this reason, the panelists should be selected for sensitivity and trained and retrained both before and during a sensory panel to maintain the acuity of each panelist. Because of the variability within and among every person, the panelist should not be interchanged during a given project. It is very important that the panelist understand the task at hand and the scales that have been assigned for use as a measurement tool. For this reason, it is imperative that a panel be trained and retrained on an ongoing basis. A panel is much like a living being within itself. A panel is made up of several human beings, but within that group of human beings, each panelist must understand and coincide with the thoughts and feelings about the samples being evaluated as every other member of that panel.

Correlation with others often proves to be a difficult task because of the everyday encounters of each panelist. Attitudes and illnesses as well as the basic human nature of perceptions will have an effect on perception. On any given day, if a panelist has had a negative encounter with a co-worker or a personal relationship preceding a scheduled sensory panel, they may have a different opinion of the samples presented than if they had just received praise from their boss or flowers from a loved one. In the same manner, if a panelist had an illness such as a respiratory infection or a headache, they may not be fully focused on performing the task at hand or more importantly, they may not be physically able to taste or smell the necessary components of a sample to fully evaluate the necessary components. Meilgaard et al. (2007) present the "chain of sensory perception" as a three-step process: (1) the stimulus hits the sense organ and is converted to a nerve signal that travels to the brain, (2) with previous experiences in memory, the brain then interprets, organizes, and interprets the incoming sensations into perceptions, and (3) a response is formulated based on the subject's perceptions. Therefore, a difference in response could be due to interference in any of the three of these steps. There could be an underlying cause such as a respiratory infection that interferes with the nerve signal to the brain. There could be a lack of sufficient information in the memory of a particular odor or taste or there could be an inability to convert the sensation into words or numbers. The use of training can be an attempt to shape the mental process so that the panelists move toward showing the same response to a given stimulus with a high repeatability (Meilgaard et al., 2007).

Meilgaard et al. (2007) report that attributes of a food item are typically perceived in the order of (1) appearance, (2) odor/aroma/fragrance, (3) consistency and texture, and (4) flavor including aromatics, chemical feeling, and taste. Without training, the subject will receive a jumble of near-simultaneous sensory impressions and will not be able to provide an independent evaluation of each (Meilgaard et al., 2007). Depending on the level of training required for specific sensory evaluation panels, each of these characteristics may or may not be addressed. Some projects require a full descriptive analysis which would require training for each area of sensory perception. However, in

some meat-science-trained panels, the first two categories are controlled as much as possible and the concentration lies within the third and fourth factors.

Appearance is often the sole influence at the retail level for a consumer purchase decision. For a meat-science application, appearance encompasses color, size, shape, and surface texture. Color and texture is influenced by a multitude of different factors (discussed elsewhere in this text). Color and texture must be accounted for in one of two ways: (1) a sensory panel evaluation of color, or (2) held constant by masking to control variation in sensory evaluation. Generally in meat-science applications, size and shape are held constant across samples.

Odor of a product is defined by Meilgaard et al. (2007) as what is detected when its volatiles enter the nasal passage and are perceived by the olfactory system. To be more specific, aroma is that of a food product and fragrances is that of a perfume or cosmetic. There are many interactions of odor with flavor. A significant amount of training must be done to create an awareness of the difference between these two sensory components.

Consistency or texture is the third set of attributes in the recognition process and often linked to those terms is velocity. These attributes are also perceived in the mouth, along with flavor. Texture can be perceived through multiple senses of sight, touch, and sound in addition to characteristics associated with eating the product. If a piece of meat is cooked rare versus if it is cooked to a well-done degree of doneness, there could be descriptors such as mushy or soft associated with sensory evaluation. Degree of doneness is only an example where texture differences may be obvious; there are many other reasons for texture differences that are discussed further in this text.

The variation in flavor of steaks and roasts may be as broad as variation in flavors across all non-meat products. The definition of flavor as written by Meilgaard et al. (2007) is that flavor which includes aromatics caused by volatile substances, the tastes caused by soluble substances in the mouth, and the chemical feeling factors that stimulate nerve ends in soft membranes. Meat flavor is related to degree of doneness, the diet that an animal was fed, the age of the meat, the age of the animal, the amount of fat in the meat, the processing factors both pre- and postharvest, and many other factors that could influence the flavor of meat. Meat flavor has been and will continue to be an area of interest for meat science for many years. There is currently an often-used lexicon created by Civille and Lyon (1996). This lexicon is widely used for training of sensory panels and is a good starting point for developing descriptive analysis ballots.

Panel Training

For a trained sensory panel, it is very important to train the panelists on how to isolate each factor and to focus on each independently of the others for evaluation of the property that is of interest to the researcher. Not only is it important to train the panelists, but it is also important to monitor the progress of each panelist throughout the process in respect to themselves and to the rest of the panel members.

Before training a panel, it is necessary to determine the need for a panel, the objectives of the project and of the panel, the personnel responsible for setup and training, the facilities, how data will be collected and handled, as well as projected costs and available funds (Meilgaard et al., 2007). After the logistics of throughput, selection and training can begin.

The American Meat Science Association (AMSA) in cooperation with the National Livestock and Meat Board published the Research Guidelines for Cookery, Sensory Evaluation and Instrumental Tenderness Measurements of Fresh Meat in 1995. This document as well as Meilgaard et al.

(2007) give a sound basis for selection and training of panel members. This text will focus on that information to describe proper selection and training techniques. There are specific test protocols such as the flavor profile, quantitative descriptive analysis, and texture profile that can be found in the ASTM Special Technical Publication 758 (1981) and the spectrum method that can be found in Meilgaard et al. (2007). This text will focus on a general model for use in most meat-science applications.

Procedures

Typical techniques for recruitment are print media and verbal communication. It must be clear in the communication that the recruitment is not for a job, but for those who are interested in food and have a genuine desire to support the panel effort. It must be clear that attendance at all scheduled trainings and the panel is essential to the success of the project, and they must be in good health. The success of the test relies on the panel member to be able to describe attributes and intensities to "tell the story" of a product. A panel member must be able to detect differences in the characteristics present and assign intensity ratings. For a true descriptive panel where the panelists come up with the lexicon, the panel member must be able to describe characteristics verbally and have the capacity for abstract reasoning.

While AMSA (1995) recommends a personal interview first, Meilgaard et al. (2007) recommend a personal interview after screening tests. To have the best panel possible, it is necessary to recruit as many people as possible to have a large pool to choose from. It would not be wise to spend a lot of time personally interviewing people if you were going to dismiss them down the road for the inability to differentiate in acuity tests. Therefore, it is wise in a large recruitment pool to use prescreen questionnaires, followed by test acuity, ranking and rating testing, and personal interviews of the remainder of the candidate pool. When a smaller number of recruits are available, personally interviewing them to get the answers to prescreening questions and determining their ability to mesh with the group dynamics would be appropriate.

For a panel of 8–10, it is generally necessary to prescreen 25–30 candidates. Information to ask for would include:

- name and contact information;
- times that they are available and for how long each time;
- if they plan to be absent during anytime during your planned range of dates for training and testing;
- any health problems such as central nervous system disorders, diabetes, high blood pressure, allergies, use of medications that might affect senses, dentures, gum disease, hypoglycemia, smoking status pregnancy or possible pregnancy during the time frame for training and testing (you must follow institutional protocol for the collection and retention of this data);
- food habit questions that would be appropriate for your situation such as likes and dislikes, restricted diets, etc.

From the pool of candidates that are not eliminated from your panel at the prescreening phase, you would next move to the acuity testing phase. To move on, the candidates should not have medical or dietary limitations, be available for training, and still be willing to move through the next phase of the selection process.

The first of the acuity testing should be detection. The detection tests should be in the form of duo–trio tests, where the candidate is either presented with a reference and then with two samples and asked which one matches the reference, or a triangle test where three samples are presented and the candidate is asked to pick out the odd one. Examples of things useful for testing are different processing times or temperatures, or a higher than normal specific ingredient level with a lower than normal level. Increase the difficulty as more samples are presented. Generally, no more than six sets of samples should be presented at a time. Candidates who respond correctly to 70–80% of the duo–trio tests or to 50–60% of the triangle tests should move to the next step.

Description testing is the second part of acuity testing. Present the candidate with products with distinct attributes such as peppermint, orange, floral, vanilla, cinnamon, etc. and have them describe the fragrance without a list to pick from. Candidates who respond correctly 80% of the time using at least a related term should move on to ranking/rating screening.

This screening will introduce the actual product that will be tested. Provide the candidate with a list of intensities with anchor points. If testing beef flavor, an 8 would be beef bouillon and a 1 would be water; for juiciness, an 8 would be watermelon and a 1 would be a cotton ball. Provide easy differences, as this is not the training phase, but simply a step to determine if they understand the task and can differentiate between two extremes on an intensity scale. It is appropriate to use the type of scale (line scale, verbal scale, or numerical scale) that will be used in the actual testing. Again, use the 80% rule and for those candidates that are left, conduct a personal interview. In the case that the personal interview is conducted before acuity screening, it is time to move to the training phase.

The purpose of the personal interview at this point is to determine if the candidate is well suited to the group dynamics. The interest in continuing should be confirmed as well as discussion of the availability to attend sessions. Very strong or very timid personalities or those who are hostile should most likely be excused as you do not want them to influence the attitudes of the other panel members.

After completion of the interviews, a mock panel should be served to assure that the panelists work well together and that they understand the process. It might be a good idea to have more people than desired during the mock panel so that there is still room for dismissal if necessary.

Remember, when excusing a candidate at any point in the process, care must be taken to avoid insulting them. Do not tell them that their answers were incorrect or dismiss them in front of the group. In a private situation, tell them that they are no longer needed and thank them for their time. One suggestion is to give each candidate a number that is private between the leader and the candidate and at the end of each session, call the numbers out that will move on to the next phase for testing.

Once the completed panel has been assembled, times should be finalized and training should ensue. Training will take between 40 and 120 h depending on the extent of the project and extent of information from the panelists desired. Training will familiarize the panelist with test procedures, improve ability to identify and recognize attributes, and improve sensitivity. It is important to stress the rules of panel etiquette during the training sessions and provide written instructions, which include:

- No eating or drinking, or chewing gum for at least 30 min before testing.
- Coffee, tea, tobacco, and other strong influences on sensory attributes should be avoided for 2 h prior.
- No perfumes or fragrances should be worn.
- Avoid talking about the samples with other panelists.

- Do not make noises during the testing such as spitting, gagging, etc.
- Instructions on handling of the sample such as consistency in the number of times chewed, if the sample should be expectorated, the time between samples, if they should take a bite of a cracker or rinse with water, etc. (Some panelists prefer using apple juice in place of the cracker and water.)

For training, written terms and definitions should be provided as well as discussed verbally. One or more sessions should be devoted to demonstrating levels of each attribute in the study. If connective tissue is of importance, steaks from animals of varying maturities or steaks from different muscles could be used. Differentiation must be made between connective tissue and myofibrillar tenderness. A good example for differentiation can be demonstrated by obtaining muscles from the longissimus dorsi of a young animal frozen in the prerigor state, which will have low connective tissue. For myofibrillar tenderness, samples can be cooked to different end point temperatures or cooked with dry versus moist heat. Low flavor intensity can be demonstrated by soaking semitendinosus muscles in water for different intervals. Flavor intensity can also be demonstrated by using steaks from veal, calf, mature bull, and mature cow carcasses. Off-flavors such as grassy, livery, fishy, grainy, etc. can be demonstrated by titrations of ingredients into ground beef. Civille and Lyon (1996) provides an extensive list of references and examples. This is also true with testing added ingredients for functionality in meats. If gums or gels or any other added ingredients (salt, phosphate, rosemary, sweeteners, etc.) are going to be tested, create titrations to train the panel with.

The panel leader should be very clear on how the samples should be handled. The number of chews, positioning on the teeth, orientation of muscle fibers, etc. must be standardized. Molars are generally used to detect firmness, compression, and rate of breakdown. Incisors relate information relative to shear, elasticity, rubberiness, and connective tissue. The tongue can also be an indicator of connective tissue by pushing it through a bolus of chewed mass.

Training will vary with different testing methods. It is never appropriate to ask a trained panel to evaluate the like or dislike of a product. If that is the desired testing, an untrained panel should be used. Trained panels are trained as testing instruments and instruments do not have an opinion. Delays or interruptions of more than 2 weeks in a series of tests should be followed by refresher training sessions and perhaps an evaluation of the panelists' performance.

Training is an ongoing process. There is day-to-day variation in the panelists and it must be monitored. Generally, a standard "warm-up" sample is served that represents the typical product being evaluated. It will assist in panelist standardization, improve concentration, increase confidence, and is a calibration among the panel.

Performance evaluation will begin soon after training starts. The leader will be able to determine if there were outliers from individual panelists. Nine samples covering the intensity scale should be spread out over 4 days with three sessions per day and three samples per session. Serving orders are rotated for each sample and each day. This can also be done with six samples over 6 days in a 6×6 Latin square. The data is analyzed as a one-way analysis of variance (ANOVA) with nine treatments and four observations per cell or a two-way ANOVA with one observation per cell. From ANOVA, the F-ratio can be calculated: F = MS treatment/MS error. This ratio shows the panelists' ability to differentiate intensity scores and their ability to repeat on the same sample a day later. The larger the F ratio, the better the panelist. Once testing begins, monitoring should be performed for each panelist and reviewed periodically.

To maintain a panel, it is important to motivate them and give them a sense that their time and effort is worth it. The panelists should be recognized publically for their contribution through announcements, acknowledgments in papers, articles, etc. Immediate gratification is generally a

good motivator. Provide a treat or if possible a monetary reward for their contribution each day. A ticket system could be used where they get their name on a ticket placed into a jar for each session they complete. At the end of the test period, a ticket is drawn and the name on the ticket wins a gift certificate or an appropriate prize. Immediate and periodic feedback is also a motivator and also an indicator of performance for the panelist and to the panel leader. Everyone has a competitive side, so provide each panelist with their own results compared with the mean of the others. They can learn from their performance this way and will strive to be closer to the mean if needed. Reward the panel at the end of the test with a lunch or dinner to celebrate the end of the testing period. A motivated panel will provide more repeatable results and will be easier to work with. Panel breakdown can occur if they are overworked, and clear boundaries on acceptable behavior is not set. Start out with a clear understanding of what is expected and how they will benefit in the end.

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12 Untrained Sensory Panels

Patricia C. Curtis

Introduction

We all have opinions about the foods we consume. Our preferences for specific foods vary by culture, geographic location, production and preparation practices, individual tastes, etc. Historically, production of high quality foods depended on a single expert in a company who evaluated the sensory properties of a food. Today, companies have come to depend on sensory panels to provide them the input they need. Trained sensory panels have replaced the single individual to evaluate quality. Trained panels provide descriptive analysis of a product. They offer no opinion on acceptance or how well a product is liked. Trained sensory panels are discussed in more detail in another chapter of this book. Untrained panels are not always able to detect the minute differences that may be detected by a trained panel. Untrained panels and consumer panels are utilized to determine how a product will be perceived in the market place. This chapter will focus on a variety of sensory tests utilizing untrained and consumer panelists. Untrained and consumer panels may be used to see if there is a perceived difference between products or to evaluate the acceptance or preference for products. Technically, these tests are generally referred to as "discrimination testing" and "acceptance or preference testing." There are a variety of ways to evaluate these perceptions. A few of the most popular methodologies will be discussed in this chapter.

Testing Locations

Untrained and consumer panels may be conducted in three types of locations: (1) in-house, (2) central location, and (3) in-home. Each type of location has its own advantages and disadvantages.

In-House Testing

In-house testing is conducted in a test facility which has a kitchen and sensory laboratory. It offers the best opportunity for control of the test. Supplies and products are readily available, as well as, facilities to prepare and serve the samples. Booths are available for the panelists and will aid in keeping the panelist focused on the task and lights may be used to mask variations between

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products. Instructions may be given to panelists as they are being served the samples. The researcher has control over the order in which the samples are presented. On the negative side, the panelist is not evaluating the product in a normal setting. Therefore, results may be biased based on how the product was consumed. For example, if the purpose of the test was to determine which steak sample (marinated vs. nonmarinated) a panelist preferred, the panelist might choose one sample if it were tasted at an in-house testing location and a different sample if it were tasted in an in-home setting. The difference in choice may be due to the way the sample was prepared (medium vs. well done) or the impact of other foods with which it was served (along vs. as part of a meal). Another bias of in-house testing could be from using employees who may have a preconceived preference related to the product.

Central Location Testing

Central locations could include shopping malls, schools, retail outlets, fairs, or even mobile labs. Central locations generally offer the opportunity to access a large number of potential panelists. Central locations offer reasonably good control. Staff is trained in product preparation and handling. It is usually easy to monitor compliance with instructions and sample presentation. On the negative side, there may be no easy way to control the flow of panelists. You may have a large number trying to participate at the same time or you may have a limited area to provide samples that does not allow for panel isolation which can lead to panel interaction during testing. Depending on where you are conducting the test, there may be limited storage area of samples.

In-Home Testing

In-home testing offers the more realistic setting for product testing. It allows panelists to try the product under normal circumstances and on multiple occasions. The results from in-home testing could provide valuable data that could support advertising claims for the product. Additional information could be obtained from family members. By allowing the panelist to use the product over a period of time, the product can be evaluated under a variety of situations (grilled, baked, fried, roasted, etc.). In-home testing provides a chance to test product preparation instructions and evaluate product and package interaction. Feedback from in-home tests can also provide critical assessment of the consumer's expectations of a product. On the negative side, the in-home test is time-consuming and costly to set up and administer. Much thought needs to be given regarding panel selection, sample size, number of products to be tested, how the products will be compared, instructions to be given to the panelists, and questionnaire development. With the in-home test, there is additional consideration of how information from different family members will be obtained and utilized. It may also be difficult to get the panelists to return the data sheets in a timely manner.

Panel Considerations

Some type of incentive is generally required to motivate a person to serve on a sensory panel. The incentive can be as simple as offering a treat (candy or snack) to the panelist at the end of an individual panel or as complex as a social event or a raffle at the end of a series of panels.

Human Subject

Researchers must be very aware of the health and safety of their panelists. The researcher should know and follow the guidelines that constrain the use of human subjects. The basis for the guidelines associated with the use of human subjects is the Nuremberg Code of Ethics in medical research (United States vs. Karl Brandt et al., 1949) and the declaration of Helsinki (Morris, 1966). Lawless and Heymann (1998) summarized the guidelines as follows:

1. It is essential that the subjects give voluntary consent to participation.
2. The subject should have the legal capacity to give the consent.
3. The subject should be able to exercise free power of choice about participating in the study.
4. The study should yield fruitful results for the good of society.
5. The researchers should protect the rights and welfare of all the subjects.
6. The researchers must ensure that the risks to the subjects associated with the study do not outweigh either the potential benefits to the participants or the expected value of the knowledge sought to society.
7. Above all, the researchers must ensure that each person participating in the study had the right of adequate consent without undue duress.

At academic institutions in the United States, all studies involving human subjects must be reviewed and approved by the institution's Institutional Review Board for the protection of human subjects in research. In commercial settings, this review is not required, but the sensory specialists should still adhere to the principles.

Recruiting Panelists

When recruiting panelists, it is important that the panelists know what is expected of them for participation in the panel and what they will receive for their participation. Panelists may be recruited from in-house or central locations, community organizations, etc. Keep in mind that panels must be recruited based on the needs to meet the objective of the sensory panel. The objective may include age, gender, cultural, or other implications that would impact the recruitment of panelists. For example, if the objective of the sensory panel was to determine the acceptability of a convenience product targeted at male college students, you would want to recruit using media that would reach that target audience.

Panel Selection

Panel selection for untrained and consumer panels will be very dependent on the objective of the sensory test. For untrained analytical (discrimination) panels, it is not really important whether or not the panel members are users of the product(s) being tested. However, consumer panel members for preference and acceptance tests should be users of the product being tested and they should represent the target populations for the study. They may or may not have good sensory function. The goal is that they represent the population of users of the product being tested. The most efficient way to screen potential panelists is to develop a screening questionnaire to address their frequency of use or consumption, degree of liking, etc. For example, if your objective was to test the preference of a

newly developed diet food which included a sugar substitute against another similar diet product, you would probably want to select the panelists on the basis of their common preference or use of similar diet foods. Selection of a panelist(s) who did not like the category of diet products being tested could provide biased results in that they dislike both products equally where panelists who normally consumed this category of diet products might find one product more appealing than the other. On the other hand, if you want to test your diet product against a nondiet product, you would not want to limit your panelist selection to only consumers who normally consumed the diet products being tested. It is also very important to ask potential panelists about their food allergies or health-related diet restrictions if the product being tested contains any potential allergens or any commonly diet restricted ingredients such as salt (high blood pressure) or sugar (diabetes). It is important to phrase selection criteria questions in a manner that they are not perceived as asking personal health-related question of the panelists. Stone and Sidel (2004) offer the following guidelines on working with test subjects:

1. Sensory skills vary from person to person.
2. Most individuals do not know what their ability is to smell, taste, or feel a product.
3. All individuals need to be instructed on how to take a test.
4. Not all individuals qualify for all tests, nor should they be expected to.
5. Subjects are rewarded for participation, not for correct scores.
6. Skills once acquired are forgotten if not used on a regular basis.
7. Skills can be overworked or fatigued.
8. A subject's performance can be influenced by numerous factors unrelated to the test or the product.
9. All subject information should be treated in a confidential manner.
10. Employees should not be paid to participate in a sensory test.
11. Test participation should always be on a voluntary basis.
12. Safety is of paramount importance and should precede all other considerations.

Panel Size and Replication

In many sensory tests, the statistical design requirements cannot be completely satisfied and compromises must be made. For example, one uses a prescreened number of panelists who met specific requirements; therefore, they are not a random selection of people. In addition, these selected panelists will most likely evaluate each product on a repeated basis and use a scale that may not be linear throughout its entire continuum and the products will not be randomly selected. However, we need to do what we can to control the variables we can control. The number of test subjects is dependent on the problem to be solved and the respective testing method. From a statistical point, difference tests should have a minimum of 20 test subjects and hedonic tests should have a minimum of 30 consumers (Jellinek, 1985; Stone and Sidel, 2004). Replication is essential for discrimination testing. Stone and Sidel (2004) recommended a single replication (each subject evaluates each product twice). In consumer testing, replication is not typical because of the more subjective nature of the judgmental process and because of the manner in which the results are used. To meet the objectives of consumer tests, large numbers of subjects are required. With a larger number of qualified (demographic and product usage) consumers, the various statistical measures become better estimates of the target population and thus the generalizations have greater validity. In consumer preference testing where "no preference" choice is given, 100 or more panelists will be needed.

Other things to consider include panelist's perceptions of color, texture, and taste. Lawless and Heymann (1998) concluded that not only are color and appearance important to the consumer in and of themselves but also that color and appearance affect the consumer's perceptions of other sensory modalities as well. Therefore, it is very important that the sensory specialists know how to ask panelists to evaluate a product's appearance and color and how to perform sensory tests to minimize the panelist's color and appearance biases from affecting the sensory results of other modalities.

The texture of an object is perceived by the senses of sight (visual texture), touch (tactile texture), and sound (auditory texture). In some products, only one of these senses may be used to perceive the product's texture and with other products a combination of these senses may be utilized. In the case of meat and meat products, texture can be extremely important to the consumer. Many surface characteristics of a food impact not only the perceived appearance of the food but also the perceived texture. Tactile texture can be affected by the sample size. Large and small sample sizes may or may not be perceived to have the same texture characteristics. Mouth-feel characteristics are tactile, but often tend to change less dynamically than most other oral tactile texture characteristics. For example, the chewiness of a piece of steak will change during mouth manipulation. Consumers often use sound as a quality indicator. For example, the "snap" when breaking a fresh potato chip.

Lighting can become an issue when visual differences are present in products being tested. In situations where color differences can be masked, colored lights may be used to mask these differences. In cases where colored lights are not sufficient to mask the differences, products can be served individually rather than simultaneously. However, a balanced serving order of products must be maintained. A balanced serving order is important because the first product in a multi-product test typically receives a higher score compared to the score it would receive in subsequent serving positions (Stone and Sidel, 2004). To minimize this impact, it is important that it has an equal opportunity to affect all products. The products must be served equally often in the first position.

It is recommended that 1, 2, 3 or a, b, c not be used as codes. According to psychologists, there is a danger of choosing sample 2 or sample b as the odd one. Psychologists, therefore, have proposed three-digit random numbers as codes (Jellinek, 1985). The number of samples served in a sensory test depends on the product being tested and the fatigue it may cause.

The testing area needs to be as free of odors as possible. This can be an issue when product samples are prepared in a kitchen adjacent to a sensory laboratory or if a test is being conducted in a central location.

Resurreccion (1998) provided the following few general guidelines for preparing meat products for sensory tests. Samples should be prepared identically. They should be similar in shape and physical condition. For example, the muscles used should be identical; the direction of the grain of the meat should be uniform. Larger samples should be cut to uniform size. The use of a template to cut the meat to the desired size is recommended. If samples are to be cut to a uniform size, odd-shaped pieces should be discarded. The cooking methods used should be identical for all samples.

From a food safety perspective USDA (2006), cooking and serving recommendations should be followed. Beef, veal, and lamb steaks, roasts, and chops should be cooked to 145°F. All cuts of pork should be cooked to 160°F. Ground beef, veal, and lamb should be cooked to 160°F. All poultry should reach a safe minimum internal temperature of 165°F. Hot food should be held at 140°F or warmer. Cold food should be held at 40°F or colder. To help keep food hot, try holding with chafing dishes, slow cookers, and warming trays. Keep food cold by nesting dishes in bowls of ice or use small serving trays and replace them often. Perishable food should not be left out more than 2 h at room temperature (1 h when the temperature is above 90°F).

Sensory Methods

The central principle for all sensory evaluation is that the testing method should be matched to the objectives of the research. The design of the sensory test involves not only the appropriate selection of the test method, but also the appropriate participants and statistical analysis. Only human sensory data provide realistic predictions for how consumers are likely to perceive food products in real life.

Discrimination Testing

Discrimination testing as a class of tests represents one of the most useful analytical tools available to the sensory specialists. Discrimination testing should be used when the sensory professional wants to determine whether or not two samples are perceived to be different. The data from a discrimination test can justify proceeding to a descriptive analysis in order to determine the basis for the difference. However, it is possible for the two samples to be different in formulation, but not have perceivable difference in the taste to the consumer. Manufacturers often conduct this type of test when they need to make an ingredient or process change in their product, but do not want the consumer to perceive a change. Discrimination testing is usually performed when there are only two samples and the difference between the products being tested is subtle. However, these subtle differences can make the risk of Type II statistical errors more likely. Roessler et al. (1978) reported that an "n" of 30 for paired tests (paired comparison and duo–trio) and for triangle tests was sufficient to produce needed significant levels. Replications can be increased by increasing the number of panelists or by having each panelist perform more tests. Ideally, the best way to increase replications is to increase the number of panelists. This is the only way to ensure each judgment is made independently. By using the same panelists to repeat their judgments on the same samples increases the possibility that the judgments are not totally independent. Discrimination test panelists do not have to like or use the product but should be capable of distinguishing between intensity levels of a given attribute. There are a number of discrimination tests available including, but not limited to triangle tests, duo–trio tests, and paired comparison test.

Triangle Tests

In the triangle test, the panelist is presented with three samples simultaneously and asked to identify the sample that is different. The test provides an answer to which sample is different, but does not provide any information about the attribute that is different. There are six possible serving combinations (AAB, ABA, BAA, BBA, BAB, ABB). The serving sequence should be randomized across the panelists with each serving order being presented an equal number of times. Statistically, this is a one-tailed test. Table 12.1 provides the minimum number of correct judgments to establish the significance at probability levels of 0.05 and 0.01 for triangle tests.

Duo–Trio Tests

The duo–trio test is useful for products with intense flavors, odors, and/or kinesthetic effects (Stone and Sidel, 2004). The duo–trio test is similar to the triangle test in that three samples are served simultaneously. However, unlike the triangle test, one of the samples is marked "reference." The

Table 12.1 Minimum numbers of correct judgments to establish significance at probability levels of 0.05 and 0.01 for triangle tests (one-tailed, $p = 1/3$)

	Probability levels	
Number of trials (n)	0.05	0.01
5	4	5
6	5	6
7	5	6
8	6	7
9	6	7
10	7	8
11	7	8
12	8	9
13	8	9
14	9	10
15	9	10
16	9	11
17	10	11
18	10	12
19	11	12
20	11	13
21	12	13
22	12	14
23	12	14
24	13	15
25	13	15
26	14	15
27	14	16
28	15	16
29	15	17
30	15	17
31	16	18
32	16	18
33	17	18
34	17	19
35	17	19
36	18	20
37	18	20
38	19	21
39	19	21
40	19	21
41	20	22
42	20	22
43	20	23
44	21	23
45	21	24
46	22	24
47	22	24
48	22	25
49	23	25
50	23	26
60	27	30
70	31	34
80	35	38
90	38	42
100	42	45

Source: Adapted from Roessler et al. (1978).
Values (X) not appearing in table may be derived from
$X = 0.4714\, z\, \sqrt{n} + [(2n+3)/6]$
where n, number of trials; X, minimum number of correct judgments; z, 1.64 ($p = 0.05$) or 2.33 ($p = 0.01$).

reference sample should be the same as one of the coded samples. The panelist is asked to pick the coded sample that is the same as the reference sample. The chance of correctly guessing the right answer is one in two. The results from the test will provide information about the perceived difference between samples, but will not provide any directional information or information about attributes that are impacted. There are two formats for duo–trio tests. Panelists will not notice any difference between the two formats, but the sensory specialist conducting the tests will see a difference in how the reference sample is utilized. Table 12.2 provides the minimum number of correct judgments to establish significance at probability levels of 0.05 and 0.01 for duo–trio tests.

Constant Duo–Trio Tests

In the constant duo–trio tests, the panelists all receive the same reference sample. This test has two possible serving orders (R_A, AB, R_A, BA) which should be equalized across the panelists. This approach seems to be more sensitive, especially if the panelists have had prior experience with the product (Mitchell, 1956). For example, if R_1 sample is the product that is currently marketed, panelists are probably already familiar with that sample. Therefore, it would make sense to have R_1 be the constant reference sample and choose the constant duo–trio test for the testing method.

Balanced Reference Duo–Trio Test

With the balanced reference duo–trio test, half the panelists receive one sample formulation and the other half of the panelists receives a second sample formulation for the reference sample. In the balanced reference duo–trio test, there are four possible serving combinations (R_A AB, R_A BA, R_B AB, R_B BA) that should be counterbalanced across the panelists. The balanced reference duo–trio should be used when both products are prototypes, unfamiliar to panelists, or when there is no sufficient quantity of product for a constant duo–trio test.

Paired Comparison Tests

The paired comparison test is a two-product test and the subject's task is to indicate the one product that has more of a selected characteristic such as tenderness, shininess, or sweetness. There are two forms of the paired comparison test: (1) directional paired comparison, and (2) difference paired comparison. The decision of about which test to use is dependent on the objective of the sensory test. If the samples to be tested are known to have a difference in a specific attribute, then the directional paired comparison test should be used. It is always more efficient and powerful to use a directional paired comparison test specifying the sensory attribute in which the samples differ, if known, than to ask the panelist to identify the sample which is different. However, it is often difficult to specify the difference or be confident that the subjects will understand or recognize the difference. By "understand," it is meant that the subjects are able to perceive that characteristic in the product. The effect of a single variable such as sweetness or saltiness cannot be totally specified. A single ingredient modification may affect many other product characteristics. Assuming that subjects are responding to only one of those characteristics is risky. If it is necessary to take the time to qualify and train the subject to recognize a specific characteristic, then the descriptive test should have been selected rather than the discrimination test (Stone and Sidel, 2004). The researcher must be sure that the two samples differ only in the single specified attribute. For both types of paired comparison tests, the probability of the selection of any one sample just by guessing is one chance in two. Table 12.2 provides the minimum number of correct judgments to establish significance at probability levels of 0.05 and 0.01 for paired comparison tests.

Table 12.2 Minimum number of correct judgments to establish significance at probability levels 0.05 and 0.01 for paired difference and duo–trio tests (one-tailed, $p = 1/2$)

	Probability levels	
Number of trials (n)	0.05	0.01
7	7	7
8	7	8
9	8	9
10	9	10
11	9	10
12	10	11
13	10	12
14	11	12
15	12	13
16	12	14
17	13	14
18	13	15
19	14	15
20	15	16
21	15	17
22	16	17
23	16	18
24	17	19
25	18	19
26	18	20
27	19	20
28	19	21
29	20	22
30	20	22
31	21	23
32	22	24
33	22	24
34	23	25
35	23	25
36	24	26
37	24	26
38	25	27
39	26	28
40	26	28
41	27	29
42	27	29
43	28	30
44	28	31
45	29	31
46	30	32
47	30	32
48	31	33
49	31	34
50	32	34
60	37	40
70	43	46
80	48	51
90	54	57
100	59	63

Source: Adapted from Roessler et al. (1978).
Values X not appearing in the table may be derived from
$X = (z\sqrt{n} + n + 1)/2$
where n, number of trials; X, minimum number of correct judgments; z, 1.64 ($p = 0.05$) or 2.33 ($p = 0.01$).

Directional Paired Comparison Test
The sensory specialist uses the directional paired comparison test to determine whether two samples differ in a specific dimension such as saltiness, texture, etc. The two samples are presented to the panelists simultaneously. The panelists are then asked to identify the sample that is higher in the specific sensory attribute. For untrained panels, it is very important that the attribute is explained in a manner that the panelists understand. The ballot to be completed should be simple. Instructions can either be given verbally or written on the ballot. There are two possible serving presentations (AB, BA). The sequences should be randomized across panelists with an equal number of panelists receiving either sample A or B first. Statistically, the test is one-tailed since the researcher knows which sample is supposed to be higher in the specified attribute.

Difference Paired Comparison Test (Simple Difference Test)
This test is best used when the product has a lingering effect or is in short supply and the presentation of three samples simultaneously is not feasible. In this approach, the sensory specialist wants to determine whether the two samples are perceived as different—no specific attribute is identified. Panelists are presented the two samples simultaneously. The panelist is asked to indicate on the ballot if the same are different. This type of test has four possible serving sequences (AA, BB, AB, BA). The sequences should be randomized across the panelists with each sequence appearing an equal number of times. Once again, this is a one-tailed test since the sensory specialists know the correct answer. The results of this will only indicate whether or not the panelists could detect a difference, but it will not provide the attribute that is different.

Ranking Tests

When using the difference test (paired, triangle, duo–trio), only two samples can be compared at the same time. In some situations, more samples need to be compared in a single test. Ranking is an extension of the paired comparison tests. Three or more coded samples are presented simultaneously. Sufficient product must be provided to allow for retesting. The number of samples tested is dependent on the panelists' memory as well as physiological considerations. With untrained panelists, no more than four to six samples should be included in a test (ASTM, 1996). Ranking forces the panelist to make a judgment between samples. Sample rankings can only provide comparisons among samples tested at any one time (Peryam and Pilgrim, 1957).

Resurreccion (1998) described several limitations to ranking. These include the fact that all products must be tested before a judgment can be made. This may result in sensory fatigue when a large number of products are evaluated and interactions occur from carryover of flavors. Untrained panelists may not understand or perceive the attribute being ranked. Finally, no indication of the absolute intensity (high or low) of the attribute being evaluated is given, and there is no measure of magnitude of difference between the products.

For ranking discrimination type properties, the sensory professional should follow the general guidelines associated with panel size, selection, etc. associated with discrimination tests. For preference or acceptability ranking tests, the sensory professional should follow the guidelines for panel size, selection, etc. associated with acceptability/preference tests.

Although test and product dependent, as a general rule to avoid fatigue, it is best to keep the sample numbers to be ranked at five or below. The ranking process is discussed in more detail along with the instruction on how results can be analyzed later in this chapter.

Acceptance and Preference Testing

Consumer acceptance and preference sensory tests are often utilized toward the end stages of product development and reformulation activities. In the early stages of reformulation and product development, there are usually too many different samples. It is toward the end of the process when the key samples are identified for consumer testing. In many cases, consumer testing is followed by market testing. The difference between consumer testing and market testing is how the samples are labeled. In consumer testing, the samples are coded, and in market testing, the tests are frequently conducted with branded products (van Trijp and Schifferstein, 1995). In consumer testing, there are two main approaches to consumer sensory testing: the measurement of preference and measurement of acceptance (Jellinek, 1964). In preference testing, the panelist makes a choice of one product over one or more other products. In acceptance testing, the panelist rates their liking for a single product on a scale. However, a more efficient method is a combination of these two procedures by determining the panelists' acceptance scores in a multiproduct test and then to determine their preference indirectly from the scores.

Preference Tests

Paired Preference Tests

Paired preference tests are a direct comparison of two products. The panelists for preference tests are generally screened to select users of the product. This test can be conducted with limited literacy panelists. It is simple to conduct and answers only one question. When panelists evaluate the product they are evaluating it as a whole. This test will not provide any answers as to why the panelists liked the product, they chose or why they disliked the product they did not choose. In fact, the panelist may like both the products and chose the one they like the most. On the other hand, the panelist may have disliked both the products and chose the one they disliked the least. To keep the statistical analysis of the results simple, the panelists should be forced to choose one of the products. Allowing the panelist to indicate they like or dislike both products equally complicates the analysis. Preference tests should not be combined with other discrimination tests because the panelists are selected differently. While panelists who are the users of the products are selected for preference test, panelists for discrimination test are selected for their ability to be analytical when evaluating the product. Discrimination test panelists do not have to like or use the product but should be capable of distinguishing between intensity levels of a given attribute.

When conducting the paired preference test, the panelist is given two samples simultaneously and asked to identify their preference. It is recommended that the panelists be forced to make a choice. There are two possible serving options (AB, BA). These serving options should be randomized across the panelists with an equal number of the panelists receiving each of the samples first. For paired preference tests, the underlying assumption is that the panelists do not have a preference for either of the products giving each product an equal chance of being chosen. Therefore, the test is two-tailed since the experimenter does not know which sample will be preferred. Table 12.3 provides the minimum number of agreeing judgments necessary to establish significance at probability levels 0.05 and 0.01.

Table 12.3 Minimum number of agreeing judgments necessary to establish significance at probability levels 0.05 and 0.01 for the paired preference test (two-tailed, $p = 1/2$)

	Probability levels	
Number of trials (n)	0.05	0.01
7	7	
8	8	8
9	8	9
10	9	10
11	10	11
12	10	11
13	11	12
14	12	13
15	12	13
16	13	14
17	14	15
18	14	15
19	15	16
20	16	17
21	16	17
22	17	18
23	17	19
24	18	19
25	19	20
26	19	20
27	20	21
28	20	22
29	21	22
30	22	23
31	22	24
32	23	24
33	23	25
34	24	25
35	25	26
36	25	27
37	26	27
38	26	28
39	27	28
40	27	29
41	28	30
42	28	30
43	29	31
44	29	31
45	30	32
46	31	33
47	31	33
48	32	34
49	32	34
50	33	35
60	39	41
70	44	47
80	50	52
90	55	58
100	61	64

Source: Adapted from Roessler et al. (1978).
Values X not appearing in the table may be derived from
$X = (z\sqrt{n} + n + 1)/2$
where n, number of trials; X, minimum number of correct judgments; z, 1.96 ($p = 0.05$) or 2.58 ($p = 0.01$).

Nonforced Preference Tests

As indicated earlier, it is much easier to statistically analyze results from preference tests if the tests force the panelists to make a choice. There is definitely a different interpretation between a test of 100 panelists of which 10 chose a "no preference" as compared to a test of 100 panelists where 75 chose "no preference." Therefore, there may be times when for one reason or another nonforced choice may be the way to go. In those situations where panelists select "no preference," there are four options in how the "no preference" ballots may be analyzed. If the "no preference" choice is used, the number of consumers answering a preference for either sample will be smaller; therefore, this option should only be used when 100 or more consumers are participating in the test (Resurreccion, 1998).

Option 1
All the "no preference" ballots can be discarded and only the ballots with a preference can be analyzed. However, this option can seriously decrease the number of usable judgments (sample tests) which will subsequently decrease the statistical power of your test results. If the "dislike both equally" choice is used, the analysis should be performed only on the preference responses (Resurreccion, 1998).

Option 2
The "no preference" ballots could be split proportionally to reflect the preference ballot results. The assumption with this approach is that the panelists who selected "no preference" would have chosen one of the samples similarly to those panelists who did make a choice. This approach is based on the research conducted by Odesky (1967) who reported the proportion of observers preferring product A or product B when "no preference" is allowed is the same as the proportion of observers preferring product A over B when a choice is forced.

Option 3
No preference ballots can be split 50:50. The assumption with this approach is that the panelists who chose the "no preference" option would have randomly chosen one product or another if they had been forced to choose. This is a conservative approach which will protect against a false positive result, but it still runs the risk of missing a significant preference.

Option 4
For paired preference studies using a large number of consumer subjects, it is possible to calculate confidence intervals based on the multinomial distribution. If the confidence intervals of the proportions of those expressing a preference do not overlap and there is a low rate of "no preference" then one could test whether one product is preferred over the other (Quesenberry and Hurst, 1964). To accurately use the multinomial distribution confidence intervals, one needs more than 100 consumer panelists with fewer than 20% of them using the "no preference" option. The formula for the confidence intervals is as follows (Lawless and Heymann, 1998):

$$\text{Confidence limits} = \frac{x^2 + 2x \pm \sqrt{x^2}[x^2 + 4x(N - x)/N]}{2(N + x^2)}$$

Where x = number of observed preference votes in a sample

N = sample size

Preference Ranking

Preference ranking is as the name implies; the panelists are asked to rank several products either in ascending or descending order according to their preference or liking. This is a forced choice in that panelists are not allowed to have ties in their ranking of products. This test is simple for the panelists and can be done with little effort. The data collected is ordinal in nature and the rank values are not independently distributed. Thus, the data are nonparametric. Preference ranking may be analyzed by using Basker tables (Basker, 1988a, 1988b). The Basker tables require that the panelists are forced to make a choice and that there are no tied rankings. To use the Basker tables, using 1 as the most preferred sample sum the rankings for each product. Using the Table 12.4, move across the table horizontally to locate the number of products tested and then move down the table vertically to locate the number of panelists. The number in the table at the horizontal and vertical intersection is the critical difference value between products rankings for significance. For example, if five products were ranked by 30 panelists, the critical difference between rankings for significance would be 33.4. In this example, the results of the sum of totals for each product are:

For Product A = 113 ab
B = 108 ab
C = 80 a
D = 140 b
E = 110 ab

Rank totals not followed by the same letter are significantly different according to this test. Product A is not significantly preferred over product B or E but is significantly preferred over D.

Acceptance Tests

Acceptance testing is a valuable and necessary component of the sensory evaluation process. In product evaluation, acceptance testing usually, but not always, follows discrimination and descriptive testing. Acceptance testing measures the liking and/or preference for one product over another. There is an obvious relationship between liking and preference. In fact, preference can be measured indirectly by comparing liking scores in a multiproduct test. It is also important to emphasize that the sensory acceptance test is neither a substitute for large-scale consumer tests nor is it a competitive alternative (Stone and Sidel, 2004).

Hedonic Scales

Of all the scales and testing methods, the 9-point hedonic scale occupies a unique niche in terms of consumer acceptability and preference testing. The most common hedonic scale is the 9-point scale. This scale measures the degree of liking. The words chosen for each scale option were based

Table 12.4 Critical values of difference between rank sums, $p < 0.05$

	Number of products								
No. of assessors	2	3	4	5	6	7	8	9	10
20	8.8	14.8	21.0	27.3	33.7	40.3	47.0	53.7	60.6
21	9.0	15.2	21.5	28.0	34.6	41.3	48.1	55.1	62.1
22	9.2	15.5	22.0	28.6	35.4	42.3	49.2	56.4	63.5
23	9.4	15.9	22.5	29.3	36.2	43.2	50.3	57.6	65.0
24	9.6	16.2	23.0	29.9	36.9	44.1	51.4	58.9	66.4
25	9.8	16.6	23.5	30.5	37.7	45.0	52.5	60.1	67.7
26	10.0	16.9	23.9	31.1	38.4	45.9	53.5	61.3	69.1
27	10.2	17.2	24.4	31.7	39.2	46.8	54.6	62.4	70.4
28	10.4	17.5	24.8	32.3	39.9	47.7	55.6	63.6	71.7
29	10.6	17.8	25.3	32.8	40.6	48.5	56.5	64.7	72.9
30	10.7	18.2	25.7	33.4	41.3	49.3	57.5	65.8	74.2
31	10.9	18.5	26.1	34.0	42.0	50.2	58.5	66.9	75.4
32	11.1	18.7	26.5	34.5	42.6	51.0	59.4	68.0	76.6
33	11.3	19.0	26.9	35.0	43.3	51.7	60.3	69.0	77.8
34	11.4	19.3	27.3	35.6	44.0	52.5	61.2	70.1	79.0
35	11.6	19.6	27.7	36.1	44.6	53.3	62.1	71.1	80.1
36	11.8	19.9	28.1	36.6	45.2	54.0	63.0	72.1	81.3
37	11.9	20.2	28.5	37.1	45.9	54.8	63.9	73.1	82.4
38	12.1	20.4	28.9	37.6	46.5	55.5	64.7	74.1	83.5
39	12.2	20.7	29.3	38.1	47.1	56.3	65.6	75.0	84.6
40	12.4	21.0	29.7	38.6	47.7	57.0	66.4	76.0	85.7
41	12.6	21.2	30.0	39.1	48.3	57.7	67.2	76.9	86.7
42	12.7	21.5	30.4	39.5	48.9	58.4	68.0	77.9	87.8
43	12.9	21.7	30.8	40.0	49.4	59.1	68.8	78.8	88.8
44	13.0	22.0	31.1	40.5	50.0	59.8	69.6	79.7	89.9
45	13.1	22.2	31.5	40.9	50.6	60.4	70.4	80.6	90.9
46	13.3	22.5	31.8	41.4	51.1	61.1	71.2	81.5	91.9
47	13.4	22.7	32.2	41.8	51.7	61.8	72.0	82.4	92.9
48	13.6	23.0	32.5	42.3	52.2	62.4	72.7	83.2	93.8
49	13.7	23.2	32.8	42.7	52.8	63.1	73.5	84.1	94.8
50	13.9	23.4	33.2	43.1	53.3	63.7	74.2	85.0	95.8
55	14.5	24.6	34.8	45.2	55.9	66.8	77.9	89.1	100.5
60	15.2	25.7	36.3	47.3	58.4	69.8	81.3	93.1	104.9
65	15.8	26.7	37.8	49.2	60.8	72.6	84.6	96.9	109.2
70	16.4	27.7	39.2	51.0	63.1	75.4	87.8	100.5	113.3
80	17.5	29.6	42.0	54.6	67.4	80.6	93.9	107.5	121.2
90	18.6	31.4	44.5	57.9	71.5	85.5	99.6	114.0	128.5
100	19.6	33.1	46.9	61.0	75.4	90.1	105.0	120.1	135.5
110	20.6	34.8	49.2	64.0	79.1	94.5	110.1	126.0	142.1
120	21.5	36.3	51.4	66.8	82.6	98.7	115.0	131.6	148.4

Source: Adapted from Basker (1988a).

on equal interval spacing (Thurstone, 1927). Wording for a typical 9-point scale for acceptance test are:

- Like extremely
- Like very much
- Like moderately
- Like slightly

- Neither like nor dislike
- Dislike slightly
- Dislike moderately
- Dislike very much
- Dislike extremely

The 9-point scale is very simple to use and easy to implement. It has been reported that the scale is reliable and has a high stability of response that is independent of region and to some extent of panel size (Lawless and Heymann, 1998). There are a number of studies where panelists were asked to indicate their responses on unstructured scales such as lines with only like and dislike anchors on each end (Lawless, 1977; Rohm and Raaber, 1991; Hough et al., 1992). Pearce et al. (1986) found that hedonic scales provide similar results to these tests in terms of reliability, precision, and discrimination. Hedonic scales have been conducted using face scales. The "smiley" faces are often used for hedonic tests with panelists who may have problems reading the descriptive anchor words, that is, non-English speaking, children, etc. However, there is some controversy regarding the effectiveness of the face scales. Kroll (1990) found no advantage for the face scales over other scales.

It is possible to convert the hedonic scale data to paired preference or rank data (Rohm and Raaber, 1991). Since the scaled acceptance data are "richer" in information, it is possible to derive these other simpler measurements from hedonic scaled data. Preference and ranking can be indirectly measured with the hedonic data.

The hedonic scales differ from other category scales in that the responses are not expected to be monotonic with increasing magnitudes of some physical characteristic, but show a peak (the maximally preferred magnitude) above and below the rating. It is possible to use the hedonic scale for magnitude estimation (Land and Shepherd, 1988). Related to the hedonic scale is the food action scale developed by Schutz (1965), where the liking categories of the hedonic scale are replaced by how often the subject would eat the food.

Summary

The discrimination and acceptance tests are powerful sensory evaluation methods in terms of sensitivity and ability to provide reliable and valid results. Stone and Sidel (2004) indicate it is important that the sensory professional recognizes the following innate qualities:

1. In practice, all discrimination tests are equally sensitive.
2. The discrimination test is a forced choice test.
3. The discrimination test usually proceeds descriptive and preference testing.
4. Products not perceived as different cannot be assigned different preferences.
5. Products perceived as different can be liked for different reasons.
6. Not all products warrant testing.

The following is a set of guidelines that are useful when planning sensory tests (Stone and Sidel, 2004):

1. Use a discrimination test only to measure whether this is a perceived difference.
2. Base selection of a particular test method on the test objective and the product's characteristics.

3. Replication is required.
4. Use a balanced serving order.
5. Do not use discrimination testing as a part of a consumer preference/acceptance test.
6. Design the test to minimize all non-product variables.
7. Avoid or minimize the use of product carriers when possible.
8. Express results from a discrimination test in probability terms.

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13 Consumer Sensory Panels

Ryan Cox

Consumer sensory tests, also referred to as affective tests (Meilgaard et al., 2007) or acceptance testing (Resurreccion, 1998) are a means by which researchers evaluate the intent of their targeted market. Thus, by its nature, it is an untrained sensory panel test. Routinely used by food companies and research groups, these tests evaluate the population for which the products are intended and are commonly preferred for product testing. More recent discussion of this type of testing has been carried out by Resurreccion (1998), Lawless and Heymann (1998), and Meilgaard et al. (2007). Consumer tests are growing in prevalence in the meat science discipline with an attempt to identify and quantify constantly evolving consumer trends and niche markets. The consumer panel's greatest shortcoming is its expense, estimated to increase at 10–15% annually (Meilgaard et al., 2007); thus, it is commonly replaced with in-house panels that are periodically calibrated against a trained panel.

The target test group for the consumer test is a representative sample of the target audience for which the product is intended. For example, less information is gained if very expensive beef cuts are tested on a group that is very conscious of cost savings. Although preference or differences may be detected, applicability of the information is lacking. Generally speaking, consumer market tests are conducted in three to four cities on at least 100–500 target consumers. Commonly, location largely determines the audience and demographic information may be collected to identify trends among factors. Testing methods may include in-house testing, retail location testing, online survey, and home use testing. Each is meant to optimize cost to the researcher while maintaining the appropriate testing environment to the product.

Although consumer tests are regularly utilized in a university research setting, private industry leads the way in the development of this type of testing. For this sector, motivation to do this type of testing typically falls into one of the following categories: product maintenance, product improvement, novel product development, assessment of market, and support for advertising or marketing claims (Meilgaard et al., 2007).

As will be discussed, consumer testing can take many forms; however, very commonly, it is carried out in a designated public venue. This allows for the greatest cross-section of the population as well as of the volume of tests performed. Generally, this type of testing has the greatest amount of variables to consider for preparation purposes. Thus, when it is not specifically stated within this chapter, this is the type of testing that will be implied.

Before delving into the theory and eventual execution of the consumer sensory test, it is first necessary to determine the entire scope of activity that this type of research involves (Fig. 13.1).

The Science of Meat Quality, First Edition. Edited by Chris R. Kerth.

Progression of Consumer Testing

- Clearly define and articulate experimental objectives
- Select and develop an appropriate test to achieve experimental objectives
- Fabricate/secure, select, and screen samples to be used
- Identify/recruit consumer pool to be tested
- Schedule and plan implementation of testing and location logistics
- Determine method of sample preparation with consideration for food safety
- Pre-testing review and possible dry-run with all research personnel
- Execute consumer testing and data collection
- Process data, analyze, and interpret results
- Report results in a timely manner

Figure 13.1 Stepwise progression of the stages of consumer sensory testing. The chapter will follow this general progression with more detail allowed for each step.

Regular review of this experimental progression by not only the project leader, but all of the research personnel associated with the project will encourage continuity and efficiency.

Developing an Experimental Approach

Acceptance versus Preference

Basically, there are two types of consumer sensory tests: the acceptance test and the preference test (Jellinek, 1964). The use of either of these methods is dependent on the type of consumer being tested and on the data that is desired. For acceptance tests, evaluation of a single sample is possible, and sometimes preferred. There is not a necessity for comparison, and evaluation is based on either a positive attitude toward a food, or actual utilization of that food by the consumer (Amerine et al., 1965). The measurement of acceptability is often inferred from scale ratings and can be characterized by the simple question, "How acceptable is this product?" (Stone and Sidel, 1993).

The preference test is performed on two or more samples on which relative preference may be determined (IFT/SED, 1981). This preference is defined as a higher degree of liking or the choice of one sample over another (Amerine et al., 1965) and its testing measures the appeal of one food or food product over another (Stone and Sidel, 1993). With this testing method, the consumer is essentially answering the question, "Which sample do you prefer?" If scaling methods are used, then the measure of preference can be implied directly from the data.

Types of Acceptance and Preference Tests

Acceptance tests are categorized to the paired preference test (subtypes that include the ranking test) and the rating test. Although other types exist, they are typically a variation of these two types of tests. For the paired preference test (Fig. 13.2), consumers are asked preference or acceptance between two samples, presented simultaneously. It is not recommended that these samples be presented sequentially, as there may be the possibility of bias based on order. The biggest advantage to this type of test is the simplicity of design and input. It does not require a great level of comprehension on the part of the consumer and may be desirable for testing with children, the illiterate, or panelists with a language barrier.

The disadvantage to the paired preference test is that it gives no indication of magnitude in either preference or dislike. Additionally, it can be considered a less efficient test because one evaluation

PANELIST_____ SAMPLE_____

Please consume both samples completely and then mark the box ☒ ☑ with the sample that you prefer. You must choose one. Please notify the staff if you need more of either sample.

8169	4073
☐	☐

THANK YOU!

Figure 13.2 Example of a paired preference survey for meat samples. Special attention is given to clear, easily readable language and concise layout. For forced decisions, there are only two possible responses. If a consumer refuses to choose in this scenario, the survey is not included with the data.

is garnered from two samples as opposed to direct evaluation of each sample. Furthermore, a "no preference" option is commonly used, and with this option, it is virtually impossible to garner any information about individual characteristics. Moreover, this type of testing may be susceptible to bias, largely unintentional, as product placement and multiple comparisons are considered.

Similar to the paired preference test is the ranking test (Fig. 13.3) that utilizes the same approach. Once again, the advantage to this type of testing is the simplicity of design and execution. It may be ideal for testing with children, the illiterate, or the linguistically challenged (Coetzee and Taylor, 1996). The data is not difficult to work with and all data are treated as ordinal. For this type of testing, the consumer is presented with three or more coded samples and asked to rank them in the order of preference. It is recommended that no more than six samples are presented at one time to account for consumer fatigue and recollection (ASTM, 1996).

As with the paired preference test, the disadvantage to this type of testing is that it provides data about a sample only in relation to other samples and not as a solitary evaluation. More simply stated, if a sample is ranked very high, this still does not necessarily correspond to a high acceptance rating. Additionally, an evaluation cannot be communicated by the consumer until all samples have been

PANELIST_____ SAMPLE_____

Please consume all samples completely and request additional sample if retasting is needed. Then place numbers on the lines (1–6) to rank the samples according to your preference. 1 = Most Preferred, 6 = Least Preferred.

704_____
426_____
619_____ NO TIES PLEASE
141_____
330_____
922_____

THANK YOU!

Figure 13.3 Example of a ranking survey for meat samples. Special attention is given to clear, easily readable language and concise layout. Researcher interaction may be necessary to ensure that no ties and all ranks and samples are represented.

tested, which may allow for the carrying over of flavors or other sensory attributes (Resurreccion, 1998). Finally, absolute intensity and magnitude of difference cannot be garnered from this type of test (Stone and Sidel, 1993). Nevertheless, if product preferences are the only information that the researcher needs, then this test may be useful and easy (Colwill, 1987).

The two most common rating tests are the Food Action Rating Scale (FACT) and the hedonic scale. The FACT scale was developed by Schutz (1965) and evaluates the acceptance of a product by a population. A measure of general attitude, the scale considers nine categories. Single or multiple samples are evaluated and presented in a balanced order. The consumer is asked to identify the statements that best describe their attitude toward the product. For statistical evaluation, ratings are converted to numerical scores.

The hedonic scale (most commonly, the nine-point hedonic scale; Fig. 13.4) is the most widely used scale in consumer testing for a number of reasons. Developed by Jones et al. (1955) and Peryam and Pilgrim (1957), the hedonic scale has been validated through research for reliability (Peryam et al., 1960; Meiselman et al., 1974; Stone and Sidel, 1993), and its ease of use makes it desirable for many applications. Alternatives to this scale have been the five-, seven- and nine-point scales and the facial scale variations of these (Fig. 13.5). A three-point scale is not recommended as adults tend to avoid extremes in evaluation. Essentially, the line scale is anchored by nine equidistant points with descriptors at each point. The middle point represents a neutral evaluation and the extremes are found at either end of the line. The methodology is easily understood by panelists and it is easy to administer.

Particularly appealing to the researcher is the utility of the data gained from a hedonic scale evaluation. Each point is considered equally spaced and thus statistically, a variety of characteristics can be calculated. The level of acceptance may actually be quantified by both mean and standard deviations. As each sample is tested individually, means may be stratified by acceptance. Responses from hedonic scales can be converted to ranks or paired preference data very simply. Parametric statistical analysis, such as analysis of variance, can provide valuable information.

Data Collection

Data collection essentially can be categorized as questioning or observation. Both methods will allow for primary data to be collected from the consumer (ASTM, 1979). With questioning, this can either be accomplished verbally or in writing. The verbal questioning method can be carried out in several ways, including over the phone or in person. If an in-person interview is not feasible or possible, often the telephone interview is conducted. The advantage to the telephone interview is that it can be conducted with in-home testing of a product. Additionally, it can be more cost effective, as there is no need for the researcher to travel. Moreover, there is little to no bias on the part of the interviewer or consumer, because a visual assessment cannot be made. Also, there is a phone number associated with each panelist, so follow-up data may be obtained or lost data may be recovered.

A disadvantage to the telephone interview is that it primarily relies on the consumer's recollection of the sensory evaluation, as the interview is often conducted after the product has been tested. Moreover, the telephone interview does not allow the researcher to evaluate or control the environment where the sensory evaluation and consumer questioning take place. Also, the researcher has less control if the consumer becomes distracted or is inclined to simply hang up the phone. Finally, there is a limitation to the representation of the random population with telephone interviews, as there is a growing number of unlisted land lines and cellular telephones are quickly becoming the primary phone for many.

PANELIST_____ SAMPLE_____

Please complete one page per sample by consuming entire sample and marking a box for each attribute

FLAVOR : Please rate the sample for flavor.

Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like Nor Dislike	Like Slightly	Like Moderately	Like Very Much	Like Extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

TENDERNESS: Please rate the sample for tenderness.

Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like Nor Dislike	Like Slightly	Like Moderately	Like Very Much	Like Extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

JUICINESS: Please rate the sample for juiciness.

Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like Nor Dislike	Like Slightly	Like Moderately	Like Very Much	Like Extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

OVERALL ACCEPTABILTY: Please rate the sample for overall acceptability.

Dislike Extremely	Dislike Very Much	Dislike Moderately	Dislike Slightly	Neither Like Nor Dislike	Like Slightly	Like Moderately	Like Very Much	Like Extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

THANK YOU!

Figure 13.4 Example of a 9-point hedonic scale survey for meat samples using text responses. Special attention is given to clear, easily readable language and concise layout.

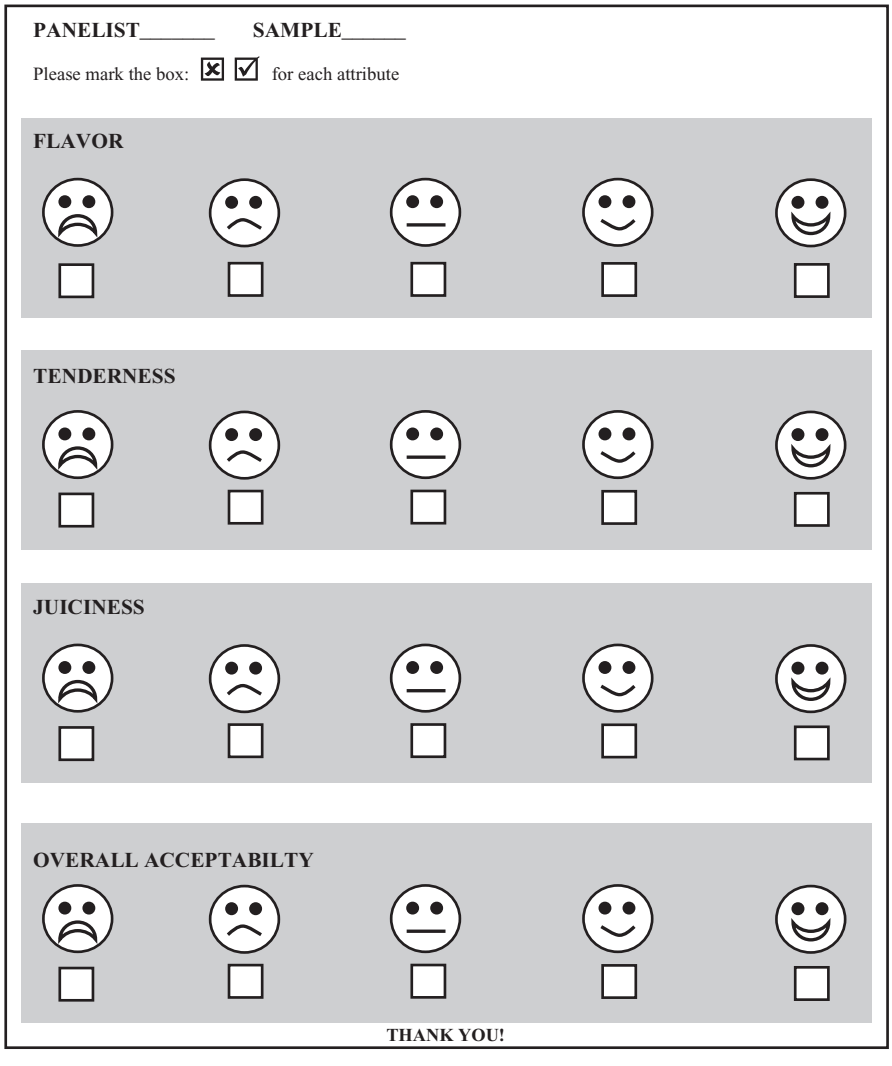

Figure 13.5 Example of a 5-point hedonic scale survey for meat samples using simple facial responses. For children, illiterate, or those with communication barriers, simplicity of design is preferred.

For the direct questioning of a consumer, or in-person interview, there are several advantages. Perhaps the greatest advantage is that a sensory evaluation can take place as the interviewer is receiving verbal feedback from the consumer. This means that the consumer is not relying on their memory to answer questions regarding their evaluation. Additionally, the interviewer will be given the freedom to use visual aids to clarify their questions, or perhaps help the consumer to respond. Also, the interviewer will be able to control and/or evaluate the testing and interview environments, as this type of questioning can take place in the laboratory, designated public setting, or the home. Furthermore, other researchers or consumers are able to observe the interview with this method for other informational purposes. Finally, the consumer is more likely to complete this type of interview, as it is very simple to hang up a telephone.

The disadvantages to the in-person questioning method include the representation of the sampling population. Like in telephone interviews, there are a limited number of consumers that will agree to an in-person interview. This may be largely due to the cost or effort of travel on the part of the researcher or consumer. Therefore, this method will also be more time consuming, and thus more demanding on the researcher and the consumer, as well as on the budget for the research. It is important for the researcher to weigh the benefits of the greater information gained from the in-person questioning method against the time, effort, and monetary cost of its use. Since budgets commonly dictate experimental design, the in-person interview is regularly abandoned for a more cost-effective alternative.

For observational data collection, there is not a necessity of direct interaction between the researcher and the consumer. The greatest advantage to this type of data collection is that the consumer will most likely not know what information is being collected from the testing, and thus, is less likely to exhibit a bias or altered behavior. This is a direct observation of a behavior, and consumer recollection or description is not necessary; thus, there is less opportunity for data alteration. Oftentimes, this method may be used as a background or "qualifying" test to determine which consumers may also be questioned verbally.

The greatest disadvantage to this type of data collection is that little or no justification may be given for an observed behavior or response. There is also no means of indicating degree of preference or dislike. Additionally, with less or no attention given to an interviewer, the consumer is more aware of the environment, and thus more likely to display bias, fatigue, or confusion. Also, the researcher will have less control of the environment due to limited or no interaction with the consumer.

The Written Survey

Several factors need to be considered when developing the question sheet that will be used in consumer sensory evaluation. It is desirable to create a positive experience that will not become a great inconvenience or burden for the consumer. With this approach, you are likely to maintain a pool of respondents for future projects. Also, an expedited survey and response process will relate to more efficient data collection.

If you plan to conduct testing within a controlled setting, such as the laboratory or the home test, you are afforded the freedom of creating a longer testing period. It is recommended that you do not exceed a 15 to 20 minute testing period, as fatigue and attention spans will not allow it. Additionally, with longer tests, it is recommended that appointments are created to ensure that no consumers are left to wait long periods of time either before or after their testing.

Depending on the pool from which you are recruiting your consumers, special attention will need to be given for testing time of day. If you are recruiting employees of your institution or company, weekday work hours are obviously preferred, with consideration given to work schedules. For the general public, it is probably more desirable to test after work hours on weekdays to allow for sufficient time to travel from the workplace. Unless interception of consumers in a high-traffic public setting is your recruiting method, it is advised that you do not test on weekends.

However, if your primary consumer pool will be in the designated public setting, utilizing high-traffic areas, there are other considerations to keep in mind. Primarily, the length of the testing time will need to be limited to less than 5 minutes. Beyond this time, the consumer will begin to feel inconvenienced and their schedules interrupted. Additionally, special care will need to be taken to ensure clarity of instructions and questionnaire structure. To meet the small time frame, repeating of instructions or further explanation is not desirable.

It is the role of the primary investigator or researcher to first determine the experimental design and testing method in order to develop the written questionnaire. There are a variety of authors (ASTM, 1979; Meilgaard et al., 2007; Resurreccion, 1998) that may be referred to for question structure and wording. Other factors to consider are the sequence of questions and the emphasis of the entire survey. To ensure that the most important information is collected from the questionnaire, include those questions first. This is to address the fact that consumer fatigue or disinterest will forbid you from collecting the most critical information. For this reason, it is recommended that all demographic information either be collected in a screening process or at the end of the primary questionnaire.

Finally, the use of open-ended, or unstructured (Resurreccion, 1998) questions is discouraged for several reasons. Literature finds support for both sides of this argument with Stone and Sidel (1993) in opposition and Meilgaard et al. (2007) in support of the open-ended question. This is any question in which the consumer is free to respond with absolutely any type or length of answer possible; a question is presented and a blank space is provided for an answer. Although this allows a larger exploration of the topic as well as a wide variety of information possibly gathered, there are a number of flaws to this type of question. From a data-collection perspective, this creates a good deal of data entry and interpretation concerns. Additionally, these responses are very difficult to quantify. Also, consumers will often not understand what type of answer is desired and will either respond with too little, too much, or no information at all.

Types of Questions

In the development of a questionnaire or survey for use in a consumer sensory panel, consideration must be given to the structure of the questions asked. Essentially, the researcher must choose between the structured and unstructured question. The structured question is the most common used in sensory surveys, and relies on scaling. This type of question is accompanied by a grouping of predetermined responses that the consumer must choose from. This methodology relies heavily on scaling, and its four categories include nominal, ordinal, interval, and ratio scales (Stone and Sidel, 1993; Resurreccion, 1998).

The nominal scale is the use of descriptive words or names for given answer categories or answers. Examples of nominal scales include “black or white,” “salt, pepper, garlic,” etc. These answers are meant to be evaluated as frequency counts, and are commonly used to classify a panelist or set of responses. The ordinal scale uses the concept of lowest to highest, described with either words or numbers. This scale is utilized to rank comparisons between different samples, and the analysis is fairly straightforward.

The interval scale, perhaps the most commonly used in sensory evaluation, particularly with hedonic scales, assumes an equal space between all points on the scale. This allows for straightforward parametric data analysis. It is considered to be a truly quantitative scale and a variety of statistical functions may be applied. The ratio scale relies largely on fixed ratios relative to a fixed point. The most common ratio scale in consumer testing is the magnitude estimation (Resurreccion, 1998).

Identifying Test Subjects

A primary step in establishing a consumer sensory panel is to identify the market for the product being tested. Often, meat products fall into very large categories of consumers, and this task may be more challenging. However, it is critical that this step take place in order to refine the pool from

Age
_____14 and below
_____15–30
_____31–40
_____41–50
_____51–60
_____61 and above

Gender
_____Female
_____Male

Annual Household Income
_____$0–$20,000
_____$20,000–$40,000
_____$40,000–$60,000
_____$60,000–$80,000
_____$80,000–$100,000
_____Over $100,000

Please place a mark next to the category that best describes you for the 3 attributes.

All information is anonymous and is only used for testing purposes.

THANK YOU!

Figure 13.6 Example of survey for general demographic data typical to consumer testing. More specific data may be collected according to experimental objectives.

which panelists may be chosen. As an example, emulsified sausages such as frankfurters tend to have a higher consumption with children, and thus it may be to the advantage of the researcher to limit the pool to this demographic.

Screening panelists is often carried out using a series of questions that identify preference markers such as age, nationality, etc.; however, the most common include frequency of use, age, gender, income, geographic location, employer, and education (Fig. 13.6). Frequency of use is of particular importance when evaluating newer products. A high frequency of use of a particular product may indicate a tendency toward trends. Inversely, a low frequency may indicate the use of a product as the best substitution for the desired product (Resurreccion, 1998).

Age of the consumer is important for the general type of product being tested. A rack of lamb roast is not tested on kindergartners, just like cartoon-themed snack foods are not tested on senior citizens. Typically, young adults receive the most consumer focus due to purchasing habits developed at this age. Additionally, this group tends to be less hindered by excessive family costs and spend more on food.

Income, often referred to as household income, is a fairly straightforward attribute that may have a role in consumer preference. Households making less than $20,000 annually are not typically the best panelists for high-end steaks, just as panelists with incomes over $150,000 annually are not appropriate for value-driven items. Although it is commonly thought that women purchase more consumer goods, the gap is actually narrowing. Evaluating a test group by gender may provide valuable insight into purchasing habits. Geographic location is very important to the screening process. It would not be appropriate to test country ham in the Pacific Northwest, just as it would not make sense to test scrapple in the Caribbean. Region does have a significant influence on preference. Finally, employer and education, commonly thought to be linked to income, do have a role in the preference that a consumer may express. Awareness of certain product characteristics due to occupation or overexposure to certain products due to occupation may influence preference.

It is important to keep in mind that meat science consumer tests usually have a broad audience and panelist screening may often be done pre- or posttesting. As subjects receive evaluation sheets, a series of demographic and background questions may be answered that allow the tester to group panelists by category for later separation and elimination by attribute. Additionally, correlation may be evaluated when all of these attributes are known.

Source of Test Subjects

Keeping in mind that the test subjects used are to be representatives of the target audience of the product, consideration needs to be given to where test subjects are recruited. More often than not, consumer sensory panelists are recruited from one's own pool of employees, recruited from the surrounding community, or are compiled of the general public. In the strictest scientific sense, it is not desirable to use employees of the company or institution conducting the test due to their inherent bias. However, as mentioned earlier, costs can become excessive for alternative types of testing and this measure may be taken to reduce cost.

In reality, employees and members of the community can often serve as excellent panelists due to their familiarity with the type of product being tested and the desired demographic. Additionally, these are people that can be recruited very quickly due to daily proximity to the researcher. The prescreening process for panelists can be much more controlled with this group and may be desirable. There are several reasons that make employees and members of the community undesirable for product testing. The very first is the same as the reason they work well: they are very familiar with the product being tested. So familiar in fact, that there may be either a negative or positive bias based on what the panelist knows of the people or person doing the testing. Also, for tests evaluating novel products, a general public perspective is far more relevant.

For a variety of reasons, the general public is a far more preferred group from which to draw panelists. It is assumed that there will be less bias based on the researcher or research group, and a certain sense of anonymity can be maintained. For the evaluation of novel products, this is the ideal audience as well. However, costs can be much higher using this demographic, especially considering the location of testing. Typically, more screening will be involved when working with this group.

However, using posttest demographic sorting, a great deal of information may also be gained from this diverse group. Furthermore, it is important to keep in mind that child and teenage panelists could be desirable, as they represent a large driver of demand on our modern food products.

Choice of Test Location

In determining the location in which testing will take place, the researcher essentially must choose among three: in the laboratory, in a designated public venue, or in the consumers' own home. Often, this decision will be largely dictated by budget; however, there are characteristics to each that may be appealing for the type of testing you are doing.

Laboratory Testing

The laboratory location obviously gives the researcher the most control of the testing environment. Testing temperature, lighting, sound, space restrictions, and a variety of other factors can be controlled to eliminate variables that may affect outcome of testing. Additionally, this location allows for the recruitment of a readily available pool of panelists, whether they are employees, students, or

other staff. Moreover, this location typically offers the lowest economic input as considerations for temporary equipment, transportation, and possible lodging do not come into play.

Public Venue Testing

The designated public venue, whether it is a store, community center, sidewalk, government building, or any other location accessible by the general public, has several advantages. This location will allow for the greatest representation of the general population for the region in which testing is done. Additionally, demographic targeting may be done to represent a sub-population. For example, if the desired testing demographic is the primary food purchaser for the household, then testing in a grocery store will allow good representation. If the desired demographic is the working adult with time constraints, a location in a commercial setting would be more desirable.

The second benefit to this location is frequency, or number of total panelists tested. Typically, location will be selected to allow exposure to the greatest number of possible panelists and more testing can be done in a given period of time. This attribute is desirable, as this location tends to also be one of the most demanding on the budget. Considerations must be given to maintaining product temperature, transportation of product and researchers, equipment (tables, electrical cords, cooking equipment), rent for the space, and lodging.

Consumer Home Testing

Finally, the home of the consumer allows for a unique testing environment that best replicates the consumers' typical preparation and eating habits. With this type of testing location, typically, the product and all evaluation surveys are given to the panelist either in the laboratory or designated public venue, and the panelist brings it to their own home to prepare at their own schedule. Obviously, this gives the researcher absolutely the least amount of control for variables that may affect testing outcome; however, this is also the location's greatest strength. This method replicates and may evaluate the method in which the product is stored, prepared, and served, and may be desirable for research involving convenience items or niche-market products. The ease or difficulty in the preparation of the product may have some effect on the overall evaluation of the product.

As for budgetary concerns, this is a widely variable category, but largely, savings can be seen as no testing location is necessary and no preparation of product takes place by the researcher. The greatest drawback to this type of testing is that a reliable panelist is difficult to identify, and product evaluations are returned at a fraction of the rate in which they were distributed. If there are steps in place to control this variability, then this location may have advantages.

Additional Considerations

Other factors may be considered when establishing the testing protocol for consumer evaluation. The time of year and day may be a factor for the evaluation. More specifically, seasonal foods such as chili or whole-roasted turkey may not receive the same evaluation in the summer months as they would in the winter months, when their consumption is more typical. This may also have an effect on a factor as simple as product temperature. Heated samples may be evaluated differently than cold samples in the summer or winter months. Additionally, the time of day must be considered. A panelist may evaluate a product differently in a time that a food is not typically consumed. Breakfast sausage links in the late afternoon may test differently than in the morning.

Checklist for Public Venue Consumer Testing

- Samples
- Cooler or refrigerator for samples
- Electric cooker
- Pans or cooking vessels
- Aluminum foil for sample storage
- Food handling implements (tongs, spatulas, etc.)
- Knives
- Cutting guide(s)
- Cutting board(s)
- Digital thermometer(s)
- Labels, placards, etc. as necessary per experimental design
- Chafing dish heaters or other heat source for temperature maintenance
- Napkins and paper towels
- Cleaning solution
- Sanitizer
- Disposable gloves
- Toothpicks
- Disposable cups
- Surveys
- Clipboards
- Pens/pencils
- Incentives
- Extension cord(s)
- Folding table(s)
- Food inspection documentation, ingredients labels, experimental protocol

Figure 13.7 Example of checklist items needed for the execution of a public venue consumer test. Additional items may be required depending on experimental design and location characteristics.

Conducting Consumer Testing

Consumer Testing Procedures

Before the consumer test is carried out, it is important that the project leader or lead researcher clearly identify or review the research objectives as well as the experimental design (Fig. 13.7). When working with larger research groups with multiple researchers and/or multiple testing locations, it is important to maintain uniformity of testing parameters so as to not introduce any outlying effects in the experiment. Additionally, an understanding of both the experimental design and procedure will make it easier for the researcher to clearly explain the procedure to the consumer.

Samples

Preparation and additional considerations specific to meat and meat quality evaluation are addressed in detail by the American Meat Science Association (AMSA, 1995). Although meat is commonly categorized the same as food products when sensory evaluation takes place, some special considerations need to be made. Most commonly, special consideration of product cooked, and storage temperature need to be closely monitored to satisfy safety concerns. Additionally, meat products tend to deteriorate at a faster rate than other foods, when exposed to extended storage times.

When conducting consumer sensory panels using meat products, it is critical to keep two factors under control: uniformity and safety. Often, meat samples are prepared in advance and are maintained throughout the testing; however, sample preparation may also take place immediately before consumption. In either case, product uniformity needs to be maintained in order to guard against bias.

With whole-muscle cuts, like steaks and roasts, as well as with ground products, degree of doneness needs to be uniform throughout the entire experiment. Often this is one of the more difficult variables to control, and it is recommended that a digital probe or thermocouple is used. Additionally, it may be necessary to have those researchers conducting the experiment run one or more "dry runs" with nonexperimental product to ensure that a uniform degree of doneness can be reached. From a food-safety perspective, it is important to remember that noninjected steaks and roasts must be cooked to a minimum of 140°F, and ground and injected products to a minimum of 160°F.

Uniformity of sample size must also be maintained. For whole-muscle cuts, it is recommended that a cutting guide be used to maintain product uniformity. It is also important in steaks and roasts to only represent lean meat that is free of connective tissue and fat. It is also recommended that testing be only done on a single muscle to maintain product uniformity. For ground products like hamburger patties, special care needs to be taken to ensure uniform thickness, as well as product size. These types of products have the tendency to crumble, so special care needs to be taken to ensure uniformity of sample size.

Finally, an adequate amount of samples need to be prepared to allow for overconsumption as well as researcher error and product degradation. For locations such as the designated public venue, where a large number of consumers will be tested, it is advised that extra samples, as much as 25%, are prepared to prevent running out without an adequate number of consumers tested.

Product Preparation

Very often, the product will need to be cooked or heated immediately before it is tested by the consumer. For the laboratory setting, this is not a great challenge, and for the home test, this is out of the researchers' control. However, the designated public venue testing location does present a unique challenge in product preparation. For cooking, it is recommended that an electrical cookery method is used; thus, electrical outlets will be necessary. Open hearth and clamshell-style grills work well for this application, and their cleaning and maintenance is simple. If a different method is used, it is important to maintain uniformity and safety. It is also important to notify relevant individuals, such as store managers or employees, that cooking will be taking place, to maintain a good relationship with the establishment in which you are testing.

For cooked products, meat samples need to be maintained at temperatures above 140°F to ensure proper food safety. Unfortunately, on overextended storage at this temperature, the product will become overcooked or dried out. Thus, it is important to keep a minimum amount of samples prepared to avoid extended storage times. Often, a chafing dish can catering heater is used to maintain temperature, but electrical food warmers can also be used.

For foods that are meant to be served cold, such as deli meats, it is important to maintain the product storage temperature below 40°F for food-safety reasons. This is not necessarily as much of a challenge because typically, product degradation is much slower in this scenario. Products, such as deli meats maintained at cold temperatures, can withstand extended storage times, and so this

allows for the preparation of a much larger amount of samples in advance and throughout testing. The temperature may be maintained by keeping sample trays on ice or in small refrigerators.

As for all other food-contact surfaces, it is critical to maintain food safety with regular cleaning and disposal of old samples. It is recommended that any food handler in the research group use disposable gloves, and that the gloves are changed regularly. All surfaces, such as cutting boards, need to be cleaned and rinsed regularly to maintain a safe preparation environment.

When testing in the public venue, it is often necessary to demonstrate to store managers or other personnel that the product that you will be testing has been either state or federally inspected. Although you will not be selling the product to the public, it is often requested as a condition of the store's permission to test. Additionally, this may be requested as part of your institutional review in the university setting.

Product Presentation and Sample Number

When presenting the product to the consumer, the researcher does not want to provide any bias by way of presentation. Product uniformity needs to be maintained throughout the testing process. Additionally, equal amounts or portions of product should be presented of each experimental treatment. An adequate amount of sample should be provided to allow for a proper evaluation of the sample; however, excessive sample may bias the test or increase the cost of the experiment. Inversely, an inadequate amount of sample will not provide for a proper evaluation of the product, and may also negatively impact all sample evaluations.

Throughout testing, it is recommended that researchers are able to demonstrate the least amount of product handling to ensure the consumer of sanitary conditions. The use of gloves and handling implements, such as tongs or spatulas, help with this. Toothpicks are a minimal cost and are regularly used for consumers to handle samples and are easily disposed of.

Execution of the Consumer Test

Throughout the consumer testing period, it is important that all parameters are uniform, and that attempts to maintain homogeneity are in place. The verbal prompts and physical factors during the test should be the same. For extended testing periods, such as the large public venue tests, or market tests, there is a probability that research personnel may change; overlap of these personnel allows for observation of testing conditions and procedures. Understanding the typical flow of traffic through a public venue will allow for a fair estimation of the amount of product needed, and cooking can accommodate this. For laboratory testing where panelists have already been recruited, it is advisable to minimize the time that each consumer must wait before each sample is given. This may have a negative effect on product evaluation.

Additionally, it may be helpful to provide a script or framework of language that can be used by research personnel, so that each consumer's experience is approximately the same. If each individual that interacts with the consumer is polite and the consumer leaves the test with a positive experience, then recruitment of future panelists will be easier.

It is expected that every consumer may not have a positive reaction to the meat samples that are being tested, or may prefer not to swallow samples. A means by which expectoration of sample can take place should be provided by the researcher. Small disposable cups work well for this use and

are not usually an excessive cost. Disposable napkins and a trash receptacle are also recommended for consumer and researcher use.

Incentives and Compensation

Depending on the project budget and the ability of the researcher to recruit consumer panelists, incentives or compensation may be considered for each consumer. For the laboratory test, where the panelists may travel a sizable distance to the testing site, it is advisable to consider some incentive. Commonly, food items such as candy or snack foods are given, but monetary incentives are also possible. The use of an incentive may ensure the researcher that the consumer may be recruited for future panels. In the public venue with large numbers of consumers, an incentive may not be possible. Often, the food item itself is enough of an incentive for the consumer to participate in the testing. All of this is also true for the home consumer test.

Data Analysis

The experimental design and type of research will dictate the statistical analysis that will be used to evaluate the data garnered from consumer testing. For further statistical evaluation of testing data, several texts are recommended (Lawless and Heymann, 1998; Resurreccion, 1998; Meilgaard et al., 2007).

It is recommended that data analysis takes place as soon as possible so that proper interpretation can occur. If small parts of surveys are not filled out, there may be the possibility of recontacting the consumer panelist if analysis takes place in a timely manner. For home consumer panels, a deadline is recommended for the return of surveys, not to exceed 3–4 weeks from the time the consumer receives the product and instructions. Excessive periods of time between the consumer interaction with the researcher and the return of the survey may cause the consumer to forget instructions or other parameters of the test.

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14 Preventing Foodborne Illness

Michael A. Davis

Introduction

Foodborne illness is an ever-increasing concern for both producers of meat and meat products, and consumers of these products. It is estimated that there are approximately 76 million cases of foodborne illness in the United States each year. Of these many cases, there are approximately 325,000 incidences of hospitalizations associated with foodborne disease and 5000 deaths (CDC, 2005). It is also estimated that foodborne illnesses cost up to $35 million in lost wages, lost worker productivity, and medical expenses.

There are many agents that are able to cause foodborne illness. However, the most commonly recognized foodborne infections are associated with bacteria and viruses. The most common bacteria associated with foodborne illness infections are *Campylobacter* spp., *Salmonella* spp., and *Escherichia coli* O157:H7. Calciviruses, or Norwalk-like viruses, are the most common type of viral foodborne illness agents (CDC, 2005). There are also many bacterial agents such as *Staphylococcus aureus* and *Clostridium perfringens* that produce toxins that, when ingested, cause foodborne illness.

Parameters that Affect Microbial Growth

Like all living species, those organisms that can cause foodborne illness require that certain parameters be met before optimal growth and reproduction can take place. Those parameters that are considered to be an inherent part of the meat or muscle tissue are referred to as intrinsic parameters, whereas those parameters that are not dependent on the substrate (i.e., meat) are referred to as extrinsic parameters (Fig. 14.1).

Intrinsic Parameters

There are six parameters for growth that are commonly referred to as intrinsic parameters:

1. pH
2. Oxidation–reduction potential (Eh)

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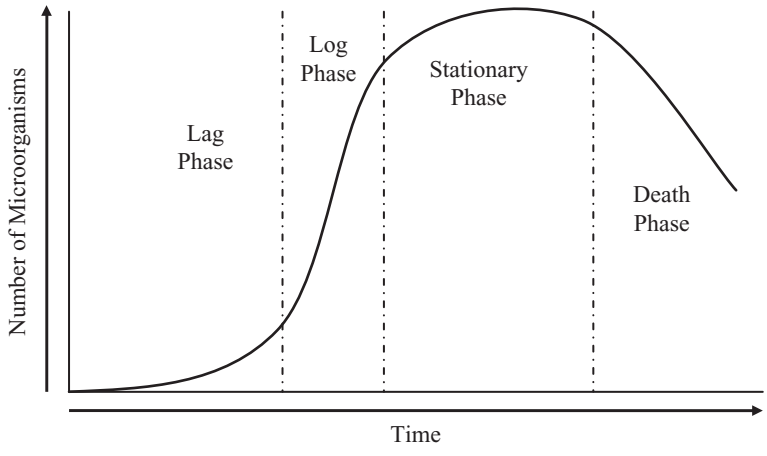

Figure 14.1 Typical growth curve of microorganisms and phases of growth.

3. Moisture content
4. Nutrient content
5. Antimicrobial factors
6. Biological structures

Each of the intrinsic factors and how they affect the growth of microorganisms on food products is discussed in the sections below.

pH

The pH of a substance is the measure of acidity or alkalinity of that substance. It is actually the p(otential) of H(ydrogen) in that substance. The scale for pH measurement is from 0 to 14 with 7 being a neutral pH. As the numbers move downward from seven, the acidity increases, while as the numbers move upward from seven, the alkalinity increases.

Most microorganisms will grow best at a neutral pH with a variance on either side of neutral of 0.5 point. Bacteria, especially those that cause foodborne illness, are very particular about the pH of a growth medium and tend to not be able to grow at pH values of less than 4.0. This is not a hard and fast rule, however, because other parameters (both intrinsic and extrinsic) can affect the minimum pH values at which some of these bacteria can grow. Although not the cause of the majority of foodborne illness cases by microorganisms, molds and yeasts can cause foodborne illness and have a greater tolerance for shifts in the pH of a substrate than do most bacteria (Table 14.1).

Most meat products attain a final pH of approximately 5.6 with variances of 0.5 point (Jay et al., 2005a) after the completion of rigor mortis. This final pH is well within the growth range of many microorganisms that can cause foodborne disease.

Oxidation–Reduction Potential (Eh)

The oxidation–reduction potential of a substance can be defined as the ability of that substance to gain or lose electrons. When a substance loses electrons, it is considered oxidized, whereas a substance that gains electrons is said to be reduced. When determining the oxidation–reduction potential of a particular substance, those that readily lose electrons are considered to be good reducing agents, while those substances that readily accept electrons are considered good oxidizing agents.

Table 14.1 Approximate minimum and maximum pH values for microorganisms commonly associated with foodborne illness (all values are approximations)

Organism	Minimum pH	Maximum pH
Fungi		
Molds	0.5	11.0
Yeasts	1.5	8.5
Bacteria		
Salmonella spp.	3.6	9.5
Acetobacter spp.	4	9.2
Listeria monocytogenes, Yersinia enterocolitica	4.2	9.5
Clostridium botulinum, Escherichia coli	4.5	9.0
Bacillus cereus, Campylobacter spp., *Clostridium perfringens, Shigella* spp., *Vibrio cholera*	5.0	9.5
Vibrio parahaemolyticus	5.0	11.0

Oxidation–reduction potential is stated in millivolts and is preceded by the term Eh. Microorganisms that are considered to be aerobic in nature must have an Eh in the positive (+) range, while anaerobic organisms must have an Eh in the negative (−) range. Most of the pathogenic bacteria that are associated with meats are considered to be aerobic. However, there are some organisms such as the *Clostridia* species that are anaerobic.

Oxidation–reduction potential for foods is determined by four characteristics:

1. The typical oxidation–reduction potential of the food.
2. The oxygen tension in the atmosphere that surrounds the food.
3. The access of the atmosphere surrounding the food to the food itself.
4. The resistance to change in the oxidation–reduction potential of the food, or poisoning capacity.

Moisture Content

The removal of moisture from a food substance or the binding of the moisture in that food substance is one of the oldest methods of food preservation. Microorganisms require water for survival, growth, and reproduction, and removing the availability of this water for biological processes is a simple way to prevent microbial growth. Water activity (a_w) is the term that is most commonly used to describe the availability of water to be used for the biological processes of microorganisms. The water activity of a substance is defined as the ratio of the water pressure of that substance to the vapor pressure of pure water at the same temperature. The equation for water activity is written as $a_w = p/p_0$, where p is the vapor pressure of the solution (substance) and p_0 is the vapor pressure of a solvent (water). The scale for water activity is 0.00–1.00 where pure water has an a_w of 1.00.

In general, most bacteria require a water activity greater than 0.90 for growth and survival with fungi having lower water activity requirements approaching 0.80. There are exceptions to this rule that include the bacterium *S. aureus*, which is able to grow and survive at a water activity of 0.86. There are also molds and yeasts that have the ability to grow where there is very low water activity (see Table 14.2). Most fresh food products have a water activity of 0.99 or greater which makes these products a good medium for microbial growth.

Reduction of the water activity of foods to certain levels will reduce the opportunity for microbial proliferation by increasing the lag phase of the typical microbial growth curve. This reduction allows for an increase in the time that passes before the final population numbers are reached and can also decrease the final population number of the microbe on the food product. It should be noted that the

Table 14.2 Approximate minimum water activity (a_w) values for microbial growth for microorganisms commonly associated with foods

Organism	Minimum a_w for growth
Spoilage microorganisms	
Bacteria	0.90
Yeasts	0.88
Molds	0.80
Microbial groups	
Halophilic bacteria	0.75
Xerophilic molds and osmophilic yeasts	0.61
Specific microorganisms	
Bacillus subtilis	0.95
Clostridium botulinum (types A and B)	0.94
Escherichia coli	0.96
Pseudomonas spp.	0.97
Staphylococcus aureus	0.86
Vibrio parahaemolyticus	0.94

water activity of a food product is influenced by other intrinsic parameters of the food, including pH, temperature, and oxidation–reduction potential.

Nutrient Content

Like all other living organisms, microbes require certain molecules and compounds that cannot be synthesized by the microbe itself and thus must be incorporated from an outside source. We refer to these molecules and compounds generally as nutrients. These include a source of energy for biological processes, a source of nitrogen, vitamins, growth factors, and minerals. Water can be included in this group but has been discussed in the Moisture Content section.

Compounds such as sugars (simple and complex), alcohols, and amino acids can be used by microorganisms for energy requirements. If a food is high in complex sugars, most microbes will break these down into simple sugars such as glucose before they are utilized. Some microbes are able to use fats as an energy source, but these types of microbes are not relatively high in numbers.

The primary nitrogen source for microorganisms on food products is amino acids. As with sugars and other compounds that are used for energy, the microbes generally utilize the least complex compounds first with some microbes having the ability to catabolize more complex compounds into simple amino acids before use.

Microbes also require vitamins, growth factors, and minerals. Many microbes are able to synthesize many of the vitamins and growth factors that are needed for growth and survival. However, most meat products have an abundance of these compounds that are available for use by the microorganisms if they cannot be synthesized.

Antimicrobial Factors

There are some foods that are naturally resistant to attack or colonization by microorganisms. These foods typically possess certain substances that are antimicrobial in nature. Most foods that have antimicrobial constituents are plants. However, there are some animal foods that contain antimicrobial factors. These include milk from cows and eggs. The majority of the meat products that are typically consumed do not possess antimicrobial factors.

Biological Structures
Many foods have natural coverings which help to prevent the entry of microorganisms. Shells, skin, and outer coverings generally help to prevent the entry of spoilage type microbes, but can provide a barrier to pathogens as well. Most meat products have the outer covering removed during processing, which removes part of the natural barrier to microbial entry.

Extrinsic Parameters

Unlike the intrinsic parameters of foods, the extrinsic parameters are not dependent on the specific food material. Extrinsic parameters of foods are the properties of the environment that the food is in. These properties will affect both the food and the ability of microbes to grow and proliferate. There are four extrinsic parameters for microbial growth on foods:

1. Product storage temperature.
2. Relative humidity.
3. Atmospheric conditions.
4. Presence of other microbes and their activity.

Product Storage Temperature
Temperatures that allow for the growth of microorganisms vary over a wide range. Some microbes can easily survive and thrive at temperatures below the freezing point of water and above its boiling point. However, most microbes of concern in meat production fall into three distinct temperature categories: *psychrotrophs*, *mesophiles*, and *thermophiles*. Temperature optimum, minimum, and maximum for these categories are found in Table 14.3. Note that these temperatures are ranges and that certain microorganisms can grow and survive at temperatures outside of these ranges.

Most spoilage microorganisms that are associated with meats are considered to be psychrotrophs, while most pathogenic bacteria are considered to be mesophiles. There are some strains of pathogenic bacteria, most notably *Listeria monocytogenes*, which are psychrotrophic and some species of *Clostridium* and *Bacillus* are considered to be thermophilic. Product storage temperature is one of the most easily manipulated extrinsic factors associated with microbial growth.

Relative Humidity
The relative humidity of the environment that the food product is stored in is of great importance when considering the possibility of microbial growth. Relative humidity of storage can adversely affect the water activity of certain foods. If a low-water-activity food is stored in an environment with a high relative humidity, water from the atmosphere can be absorbed into the food, thus allowing the microbes the ability to grow and proliferate. In contrast, foods that normally have a high water activity will decrease their surface water activity when stored in an environment with a low relative humidity. This process, although not typically important in microbial growth, can lead to the food

Table 14.3 Optimum, minimum, and maximum temperature ranges (°C) for psychrotrophs, mesophiles, and thermophiles

Category	Optimum	Minimum	Maximum
Psychrotrophs	20–27	−10 to 8	32–43
Mesophiles	30–40	20–40	40–45
Thermophiles	55–65	40–45	>65

product obtaining undesirable quality characteristics. Relative humidity is also important when combined with temperature in products with wrapping of products with a natural covering such as eggs. In these cases, temperature abuse combined with a high relative humidity can cause surface moisture, which can have microbes contained within it, to be drawn into the product, thus allowing further contamination.

Atmospheric Conditions
One of the major intrinsic factors that affect microbial growth is the oxidation–reduction potential. This potential can be affected by the extrinsic factors of atmospheric conditions, or the presence (or absence) of certain gases in the environment. The presence of oxygen in the atmosphere can certainly impact the ability of anaerobic microorganisms to grow and proliferate. Also, certain modified atmospheres, such as ozone or carbon dioxide that are used in packaging, can inhibit the growth of certain microbes.

Presence of Other Microbes and Their Activity
The biota on a food or meat product is complex, typically consisting of many different types of microorganisms that range from spoilage organisms to pathogens. Some microbes have evolved the ability to produce certain substances that will be either inhibitory or lethal to other microbes. Examples of these substances include bacteriocins, hydrogen peroxide and derivatives, antibiotic compounds, and organic acids. The production of these substances can dramatically alter the microflora present on a food compound. Microbes also compete with each other for essential substances such as water and nutrients. Combined with storage temperature, relative humidity, and other factors, one type of microorganism may out-compete others for essential substances. This process is typically referred to as competitive exclusion.

Prevention of Foodborne Illness—Processing Operations and Management Tools

Before specific strategies can be employed for detecting and reducing or eliminating specific pathogens, an overall assessment of the processing operation should take place. This overall assessment should start with general strategies and progress to the point where specific strategies can be used. There are three programs that are typically assessed in general processing operations: Good Manufacturing Practices (GMPs), Hazard Analysis and Critical Control Point (HACCP) programs, and Food Safety Objectives (FSOs).

Good Manufacturing Practices

As the science of food safety has evolved, it has become apparent that targeted programs such as HACCP and FSOs must be supported by facility-wide prerequisite programs that focus on sanitation and food hygiene. These programs are generally referred to as Good Manufacturing Practices or GMPs. According to the National Advisory Committee on Microbiological Criteria for Foods (NACMCF, 1998):

> Each segment of the food industry must provide conditions to protect food while it is under their [sic] control. These conditions are prerequisite to the development and implementation of HACCP. . . . Prerequisite programs provide the basic environment and operating conditions that are necessary for the production of safe, wholesome food.

These GMPs are not considered to be a formal part of the HACCP system or FSO, but they are considered to be crucial since they are facility-wide, not product-specific, and usually cross all product lines within a processing plant. Adequate and well-maintained GMPs will allow the processor to have more manageable targeted programs, while the absence of GMPs can lead to more complex targeted programs. All GMPs should be based on written procedures and have assigned responsibilities. These assigned responsibilities should include measurement criteria, record keeping, and corrective actions. Since GMPs are typically facility-wide, they may cross many departments and product lines. Therefore, it is important to manage the programs efficiently. Specific GMP topics are described in the following sections.

Facilities and Premises

Overall, meat-processing plants should be located, designed, constructed, and maintained with hygienic principles in mind. This includes an adequate pest control strategy since many microorganisms can be vectored into the processing operation via pests. Waste collection areas and other harborages for pests including decorative plant species should be placed in areas that do not readily allow access to the plant and kept to a minimum. Product flow and employee traffic patterns should be a major consideration when designing the facility to make sure that product flows easily through the plant. Facility design should also incorporate allowances for sufficient lighting, ventilation, and storage space. The interior design should incorporate walls, ceilings, doors, and other structures that are easily cleaned and sanitized. Special consideration should also be given to the amounts of water that are used in meat-processing operations when choosing construction materials.

Sanitation

Effective sanitation helps to reduce the microbial load in a processing area by elimination of nutrients and available water along with the use of some antimicrobial compounds. Product contact-surface sanitation is crucial to prevent cross-contamination. USDA-FSIS regulations (9 CFR 416) direct that each processing plant must have Sanitation Standard Operating Procedures (SSOPs) that are adhered to during daily processing operations. These SSOPs cover all areas of the plant, including overall plant sanitation and product contact surfaces including equipment and utensils used in daily operations. These SSOPs should have a daily written record and address areas of corrective action and product disposition if a deficiency is found. Ineffective or otherwise incomplete SSOPs can result in a noncompliance report being issued by USDA-FSIS.

Equipment

Equipment used in meat processing should have an overall sanitary design. The design of equipment should ensure that it is not a factor in direct product contamination. The equipment should be made of materials that are nonreactive with substances that will be used in the processing area and it should be easy to maintain, clean, and sanitize. All mechanical equipment will require preventive maintenance and repair over the lifetime of the equipment. These procedures should be carried out in such a way that it does not promote product contamination. Records of maintenance or repair should be kept with the equipment SSOP.

Incoming Material

All meat processors will receive materials other than the raw product into the plant that are essential to manufacturing the finished product. These materials include, but are not limited to, ingredients, packaging, personnel attire, sanitizing agents, and other chemicals. All suppliers of incoming materials should have safety and SSOP programs in place. Some suppliers may also have HACCP

plans in place. These programs should be verified by qualified personnel. Written verification from suppliers is also recommended in the form of Letters of Guarantee or Certificates of Analysis. Processors should have written SOPs for incoming materials and their storage.

Employees and Personnel
Both current and new incoming employees should receive training, not only in current processing operations and the requirements of their position, but also in the role that processing plant personnel have in the safety and quality of the food supply. Topics that should be covered include current food hygiene practices, personal hygiene, and avoidance of product contamination.

Processing Operations
Most processing operations that are included in GMPs are included for quality control. In many cases, quality control issues are also related to food safety. Examples of these types of operations include ingredient mixtures and cut-up operations. Ingredients must be mixed properly according to manufacturer's recommendations to produce a quality product. However, some ingredients may be restricted in their use or may pose some other type of safety hazard if used incorrectly. Meat cut-up is also important to final product quality, but if performed incorrectly or without proper controls, physical safety hazards such as bone or metal may become embedded in the product. Product handling and disposition instructions can also be included in GMPs.

Pest Control
Pest control in the processing area is of major importance. Pests such as insects, rodents, and birds can be vectors for disease and may also cause general unsanitary conditions within the plant. Many operations contract pest control with an outside agency. These agencies must be fully aware of the types of products manufactured so that no unapproved pest control measures will be taken. General housekeeping and sanitary operations will help to reduce the overall incidence of pests in the processing area, but management should have a proactive approach to pest management in general.

Hazard Analysis and Critical Control Points

This section offers an overview of HACCP and the principles behind the system. To establish a formal HACCP system for a food product, a more comprehensive reference should be consulted. Some possible references include:

- Corlett (1998);
- ICMSF (1988);
- Stevenson and Bernard (1995).

HACCP is a scientific and systematic approach in the production of safe food products (Pierson and Corlett, 1992; Stevenson and Bernard, 1999). The HACCP system is considered to be a management tool which focuses on the planning, control, and documentation of the production of safe products. While GMPs are typically facility-wide in program construction and focuses on quality and some safety, HACCP is product specific and focuses only on food safety. There are

seven principles that define the HACCP system as it is used in food processing operations today (NACMCF, 1998):

1. Identification and assessment of food safety hazards.
2. Identification of critical control points (CCPs).
3. Establishment of critical limits for CCPs.
4. Establishment of CCP-monitoring procedures.
5. Establishment of corrective actions for deviations.
6. Establishment of validation and verification activities and procedures.
7. Establishment of record-keeping procedures.

To prepare a HACCP plan for a processing operation, management must first identify a HACCP team. These teams usually consist of processing supervisors, quality and safety personnel, unit managers, and some hourly employees. The first objective is to define each product that needs to have a HACCP plan. Since HACCP plans are specific to products, a plant may have multiple HACCP plans in place. After products are identified, each step in the process of production of that product is identified. Once identification of processing steps has occurred, the HACCP team must determine all potential food safety hazards that can occur at each step. Hazards are typically identified by how the hazard occurs, that is, biological hazards (microorganisms), chemical hazards (food chemicals, additives, etc.), or physical hazards (bone, metal, etc.). After identification of all potential hazards has occurred, the hazards are ranked according to the likelihood of occurrence or by the severity of the illness. For hazards that have a high likelihood of occurrence or a high severity of illness, CCPs are identified. Specific limits are then placed upon the CCPs. These limits must be scientifically valid for control of the hazard to which they are attached. Once the CCPs have been identified and critical limits set, monitoring procedures for each CCP are assigned. These monitoring criteria must also be scientifically valid to ensure that the hazards are under control. If the critical limits for a CCP are not met, then a deviation is considered to have occurred. The HACCP plan must also include corrective actions that are to be taken in the event that a deviation does occur. These corrective actions should include steps to bring the process back under control (i.e., back within the critical limits) and identify the disposition of the product that was produced while the deviation occurred. Finally, records must be kept of all CCPs and deviations so that the overall reliability and validation of the HACCP plan can occur.

Food Safety Objectives and Performance Objectives

A food safety objective (FSO) is defined as "the maximum frequency and/or concentration of a hazard in a food at the time of consumption that provides or contributes to the appropriate level of protection (ALOP)" (ICMSF, 2006). As defined in this context, the appropriate level of protection is the level of risk that a society is willing to accept. The concept of FSOs is to convert this risk that the public is willing to accept into a quantifiable target for food producers. The FSO itself only sets the target for the food producers to achieve. It is the responsibility of the producer to determine the method of the achievement, if that method is scientifically valid and if the method is acceptable to consumers.

FSOs are set by governing bodies or agencies. FSOs are only used in situations where the limit set will have an impact on public safety, and may not be used for all food products. These agencies may

set the level of an FSO to pertain to any process in the production of the food product, but many set levels for foods "at time of consumption" by the consumer. An example of the type of verbiage that may be used is that a particular microorganism such as *L. monocytogenes* be absent from a serving of food at the time of consumption. This type of FSO cannot always be clearly defined for certain types of foods, especially if the production of these foods does not involve a lethality process step such as cooking. For these situations, processors typically set a performance objective (PO) from the FSO that can be achieved in the processing plant. This can be achieved by setting percentage limits or other quantifiable limits that must be met by the food processor to meet the FSO.

POs may also be set by governing bodies in the absence of an FSO. This usually occurs when the governing body views raw foods as potential cross-contamination sources for other foods. In these cases, a PO may be set in an early part of the processing chain to help deter cross-contamination further down the processing chain.

In many instances, microbiological criteria (MC) are used to measure compliance with an FSO or PO. In these cases, the microbiological data must be presented along with the food product type, the sampling method that was used in the collection of the data, and the microbiological standard that is to be met. MC have traditionally been used to test shipment lots for acceptance or rejection if there is limited or no prior knowledge of the processing conditions that the product was produced under. With the use of FSOs and POs, MC can be used to determine compliance.

Finally, it is important to recognize that FSOs and resulting POs must be technologically feasible. Many consumers would like for the food supply to be free of all possible contamination, but with current food processing technologies, this is unachievable. Governments and agencies must seriously consider all aspects of any food safety problem before introducing an FSO or PO that cannot be reasonably met by food producers. It is also important to understand that food and meat processing is a dynamic industry that constantly undergoes changes. The use of FSOs and POs is only the latest step in process of continuing food safety.

Chemical Food Protection and Preservation

One of the most common methods of food protection involves the use of chemical agents during processing operations. Many different chemicals have been investigated for their action as preservatives, sanitizers, or as antimicrobial agents. Not all chemicals reviewed in this section are appropriate for all types of meat production. Before the use or implementation of any chemical in the processing area, consult current federal regulations.

Chemical Sanitizing Agents

Chlorine and Chlorine Compounds

Chlorine is one of the most common antimicrobial agents used in food processing. Chlorine has been used effectively to sanitize drinking water and has a history of use on food contact surfaces. The Chlorine used in processing operations typically comes from chlorine compounds such as calcium chloride, sodium chlorite, and sodium hypochlorite. Chlorine is commonly used in concentrations at or below 200 ppm. Chlorine efficacy is affected by many factors in the processing plant, including initial microbial concentration and organic load, temperature, pH, and water hardness. Concerns about the use of chlorine include the buildup of organochlorides from the combination of chlorine

with amino acids. Amino acid reactions with chlorine increase as the pH increases up to pH 9. Chlorine in its traditional form can also corrode equipment.

Chlorine dioxide is also used as a chemical sanitizer in place of traditional chlorine. Chlorine dioxide tends to have a greater efficacy than traditional chlorine in the presence of high organic matter loads. Chlorine dioxide is also more effective than traditional chlorine over a wider pH range and does not typically react with amino acids, thus reducing the possibility of organochloride formation.

Electrolyzed Water

Electrolyzed water, or electrolyzed oxidizing water, has been shown to be effective at reducing bacterial loads. Electrolyzed water is produced in an apparatus that contains regular tap water and common table salt (NaCl). The water and salt in this machine are separated by a membrane. When current is applied in this machine, chemical reactions take place that result in products on the cathode side having a pH around 11 and an oxidation–reduction potential close to −800 mV. On the anode side, the substance that is formed has a pH around 2.5 and an oxidation–reduction potential over +1100 mV. This acidic water is more antimicrobial than the basic water derived from the cathode. The acidic water may also contain free chlorine up to 80 ppm and hypochlorous acid molecules. The antimicrobial properties of electrolyzed water are mainly attributed to the extremes in the oxidation–reduction potential with added benefits of chlorine and hypochlorous acid in the acidic water from the anode. Electrolyzed water production is shown in Figure 14.2

Ozone

Ozone is an oxidizing agent like chlorine but is more powerful than chlorine. Ozone can be used either in solution or in its gaseous state. Ozone has been used since the early 1900s as a disinfectant for wastewater plants to reduce color, odor, and turbidity (Conner et al., 2001). Like chlorine, ozone's efficacy is reduced by high loads of organic matter and it is corrosive to certain types of equipment. Ozone is considered generally recognized as safe (GRAS) for many foods, but because of its strong oxidizing power, it is not recommended for use in red meats. Ozone's efficacy in poultry chiller water and on poultry carcasses was evaluated by Sheldon and Brown (1986). Over 99% of microorganisms washed from poultry carcasses were destroyed by the use of ozone. In this study, there was also a decrease of 30% in chemical oxygen demand in the chiller water without any significant skin color issues, lipid oxidation, or off-flavors resulting from the use of ozone.

Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a weak acidic compound that is made in some capacity by all aerobic organisms. It is degraded by the enzyme catalase to form water and oxygen. Hydrogen peroxide is used on some food contact surfaces but is not deemed acceptable for use by the US Food and Drug Administration except when used in combination with acetic acid to form peroxyacetic acid (Jay et al., 2005b). Hydrogen peroxide has been investigated for use in poultry operations for its bactericidal activity. However, carcasses that were chilled in hydrogen peroxide exhibited a bloated skin appearance with gas and water accumulation under the skin and bleached and rubbery skin texture (Lillard and Thomson, 1983; Izat et al., 1989).

Acidified Sodium Chlorite

Acidified sodium chlorite, or ASC, is marketed by Alcide Corporation (Redmond, WA, USA) and has previously been marketed under the product name The Sanova Food Quality System® (Conner et al., 2001). This chemical is a mixture of either citric or phosphoric acid and sodium chloride

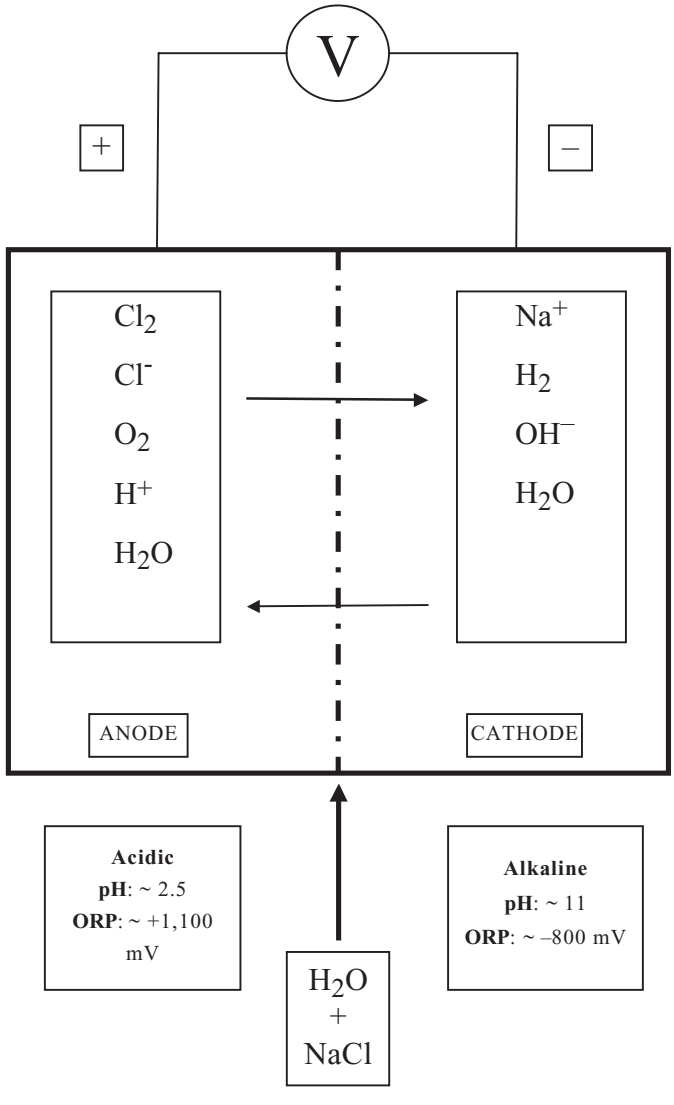

Figure 14.2 Diagram of electrolyzed water production.

(NaCl). The antimicrobial action of ASC is the nonspecific disruption of oxidative bonds on the cell membrane surface by the dissociation of the chlorite molecule (Kemp et al., 2000). This compound has been approved by the FDA for use as a sanitizer on most meat surfaces including poultry, red meat, and seafood. ASC is typically used as either a spray or a dip. This product has also been approved for online poultry reprocessing by USDA-FSIS.

Cetylpyridinium Chloride

Cetylpyridinium chloride (1-hexadecylpridinium chloride), or CPC, is a quaternary ammonium compound that has been previously approved for use in mouthwashes to prevent dental plaque. CPC operates at a neutral pH and is considered a cationic surfactant. The cetylpyridium ions interact with

acid groups on bacteria. These interactions cause the formation of weak ionized compounds that interfere with and inhibit bacterial metabolism (Conner et al., 2001; Jay et al., 2005b).

Chemical Preservative Agents

The Chemical Sanitizing Agents section of this chapter described various chemical food sanitizing agents. In general, sanitizing agents are effective against pathogenic organisms but they may also be effective against spoilage organisms. In contrast, most chemical preservatives are used for their ability to extend the shelf life of certain foods. It should be noted that some sanitizers may act as preservatives by destroying or inhibiting spoilage organisms whereas some chemicals that are classified as preservatives may also function as a sanitizer. An example of this would be the use of nitrites to inhibit *Clostridia* species (Table 14.4).

Organic Acids (Acetic and Lactic)

Acetic and lactic acids are two of the most common organic acids used in foods today. In most cases, the production of these acids is accomplished by the use of acetic- or lactic acid–producing bacteria in the production of the food. Typically, the antimicrobial properties exhibited are due to the increase in acid production by the bacteria and the resultant lowering of the pH in the food. These acids have also been shown to express metabolic inhibition in some bacterial species by the undissociated acid molecule. Lactic acid has also been shown to cause changes in the permeability of the outer cell membrane in some Gram-negative bacteria. This action may allow lactic acid to act as a mediator to other antimicrobial compounds (Alakomi et al., 2000). Bacteria that have been shown to be susceptible to organic acids include the salmonellae and other enterobacteracea.

Sorbic Acid/Sorbates

Sorbic acid is commonly used as a food preservative in its salt form as calcium sorbate, sodium sorbate, or potassium sorbate. Sorbates are commonly used to inhibit the growth of molds and some yeasts and work best when the pH is below 6.0. However, sorbates have also been recognized to possess antibacterial properties. Bacteria which are catalase positive tend to be more sensitive to the action of sorbates than those which are catalase negative. This is also true for aerobic microorganisms versus anaerobic microbes. A short list of bacteria that are sensitive to sorbates includes the salmonellae, many coliforms, and *S. aureus*. Another interesting note about sorbates is that lactic acid–producing bacteria tend to be resistant to sorbates when the pH is above 4.5. This allows sorbates to be used for their antifungal and antibacterial properties in foods that undergo fermentation by lactic acid bacteria (Jay et al., 2005b).

Table 14.4 Common chemical food preservatives

Preservative	Maximum tolerance	Organisms affected
Sorbic acid/sorbates	0.2%	Molds
Sulfur dioxide/sulfites	200–300 ppm	Insects and microorganisms
Propionic acid/propionates	0.32%	Molds
Sodium nitrite	120 ppm	Clostridia
Benzoic acid/benzoates	0.1%	Yeasts and molds

Sulfites and Sulfur Dioxide
Like the sorbates, the sulfites that are typically used in food processing are either the sodium or potassium salts of sulfite, bisulfite, or metabisulfite ($–SO_3$, $–HSO_3$, and $–S_2O_5$, respectively). These salts along with sulfur dioxide are considered GRAS for use in foods up to levels of 200–300 ppm. The actual mechanism of sulfur dioxide and the sulfites is not known but these compounds have been shown to be effective against organisms such as *Acetobacter* spp., *Clostridium botulinum*, and *Saccharomyces*.

Propionic Acid/Propionates
The propionates are the calcium and sodium salts of propionic acid. Propionic acid is a short three-carbon organic acid and is a much smaller molecule than sorbic acid. The propionates are used for mold inhibition in certain foods and tend to have the best action when the pH is above 6.0. Propionic acid seems to have the same mode of action as sorbic acid and benzoic acid. The details of this action will not be discussed here, but the overall result is that amino acid transport across the cell membrane and within the cell structure of the target organisms is affected unfavorably.

Nitrites and Nitrates
The nitrates and nitrites commonly used in curing salts for meat products are the sodium salts of molecules, sodium nitrate ($NaNO_3$), and sodium nitrite ($NaNO_2$). These compounds are used for their inhibition of microorganisms, both spoilage and pathogenic, and for their stabilization effect on red meat color. Of the two, the nitrite molecule is of more importance because of its highly reactive nature. Nitrite can be either a reducing or an oxidizing agent with the common endpoint molecule being nitric oxide (NO) which is the major molecule for color fixation in red meats.

The main microorganism of concern that is inhibited by these compounds is *C. botulinum*, although other *Clostridia*, including *C. perfringens*, are inhibited as well. It should also be noted that the addition of nitrite culture media before autoclaving results in the production of an agent referred to as the Perigo Factor (Perigo et al., 1968; Perigo and Roberts, 1967). Results of an experiment involving the addition of nitrite before heating concluded that ten times less nitrite was needed to reach the same inhibition threshold than if the nitrite was added after heating (Jay et al., 2005b). Although the efficacy of this method has been questioned for use in meat products, its efficacy in culture media was demonstrable.

Benzoic Acid
Benzoic acid was the first chemical preservative approved for use in foods by the FDA (Jay et al., 2005b). This molecule is also used by food producers for its inhibition of yeasts and molds. Like sorbic acid and others, the efficacy of benzoic acid is highest in foods that exhibit a low pH. The mode of action of benzoic acid against microorganisms seems to be its ability to prevent the uptake of certain molecules and its ability to prevent the oxidation of certain compounds such as glucose and pyruvate (Freese et al., 1973).

Other Chemical Preservatives

Ethanol
Ethyl alcohol, or ethanol (C_2H_6O), is not usually added to food items directly but is found in many flavoring extracts. Ethanol's antimicrobial properties stem from its properties as a desiccant and from its denaturing properties. The denaturing properties of ethanol seem to come from its ability

to change the permeability of the cell membrane. This change allows the cells to become vulnerable to other antimicrobial agents. Ethanol also seems to sensitize some microbes to lower pH, such as *L. monocytogenes*.

Chitosans

Chitosans are made from chitin, a long-chain polymer of N-acetylglucosamine, which, in turn, is a derivative of glucose. The chitosans are cationic polysaccharides produced by acid hydrolysis or enzymatic hydrolysis of chitin. The mechanism of chitosans involves the cationic binding to negatively charged bacterial cells. This binding interferes with cell membrane function and with membrane transport capabilities of the cell. These molecules are variable in size and are more effective against Gram-positive bacteria rather than Gram-negative bacteria. The chitosans are currently being researched as possible antimicrobial compounds for use in packaging films. Chitosans have been shown to be effective at inhibiting bacteria such as *Bacillus cereus*, *L. Monocytogenes*, and *Salmonella enterica* serotype Typhimurium (No et al., 2002; Zivanovic et al., 2004).

Indirect Antimicrobial Chemicals

The chemical additives covered in this section are primarily added to foods for results other than antimicrobial activity. However, many of these chemicals also possess some antimicrobial properties against bacteria, fungi protozoa, viruses, and mycoplasmas (Branen et al., 1980; Fung et al., 1985). Many of these chemicals exhibit their antimicrobial activities when combined with other antimicrobial agents. A partial list of these chemicals, the organisms affected and chemical's primary use can be found in Table 14.5.

Flavoring Compounds

There are many flavoring compounds currently available that possess a degree of antimicrobial activity. Generally, the compounds that are used for flavoring tend to be active against fungi more than against bacteria. In one study (Jay and Rivers, 1984) of flavoring compounds, almost one-half of the 21 compounds studied exhibited a minimum inhibitory concentration of 1000 ppm or less against both fungi and bacteria. Jay (1982) also reported that diacetyl is an effective antimicrobial. Diacetyl is often added to foods to deliver the aroma of butter. An interesting property of diacetyl is that unlike other compounds, it tends to have a greater antimicrobial effect on Gram-negative bacteria and fungi than on Gram-positive bacteria. Jack et al. (1995) found that in plate count agar

Table 14.5 Common indirect antimicrobial chemicals

Chemical	Organisms affected	Main use
Phosphates	Bacteria	Water binding, flavoring
Lauric acid	Gram-positive bacteria	Defoaming agent
Diacetyl	Gram-negative bacteria, fungi	Flavoring
Spices	Bacteria, fungi	Flavoring
BHA (butylated hydroxyanisole)	Bacteria	Antioxidant
BHT (butylated hydroxytoluene)	Bacteria, viruses, fungi	Antioxidant
Sodium citrate	Bacteria	Buffer
Monolaurin	Gram-positive bacteria, yeasts	Emulsifier

at 30°C and pH 6, 24 of 25 Gram-negative bacteria and 15 of 16 yeasts tested were inhibited by concentrations of 300 ppm diacetyl.

Antioxidant Compounds

Antioxidants are added to food products to prevent oxidation of fatty acids and lipids. While some meat products contain proportionately more saturated fatty acids and lipids, oxidation of unsaturated lipid can lead to off flavors that the consumer will find unacceptable.

The antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *t*-butylhydroxyquinoline (TBHQ) have exhibited inhibition to both Gram-positive and Gram-negative bacteria along with yeasts and molds at strengths of 10–1000 ppm. Some typical foodborne pathogenic bacteria that are inhibited by antioxidants include salmonellae, *S. aureus*, *B. cereus*, and *Vibrio parahaemolyicus*. These pathogens have been shown to be inhibited at concentrations less than 500 ppm (Jay et al., 2005b).

Phosphates

The phosphates are phosphorus salts that are added to many processed meats to increase their water-holding capacity. Phosphate compounds range from simple compounds with one phosphate up to very complex molecules with as many as 13 or more phosphorus atoms in the molecule. Phosphates have also been shown to add flavor to foods and possess some antioxidant properties. One of the most common phosphates used today in meat production is trisodium phosphate. This compound is regularly used in poultry processing as an antimicrobial aid for online reprocessing of carcasses.

Other Indirect Antimicrobial Chemicals

There are also other chemical compounds that are added to foods that possess a degree of antimicrobial activity. These include spices and essential oils along with medium-chain fatty acids and esters of these acids. Because the addition of these chemicals varies widely with different foods, a full discussion of methods and actions will not be covered. Many of these substances also exhibit their activity in conjunction with other chemicals.

Competitive Exclusion and Interference

Competitive exclusion and microbial interference are two terms that are commonly used interchangeably. However, there is a difference between the concepts. Competitive exclusion is a type of microbial or bacterial interference. Microbial interference is the prevention of microbial growth or the destruction of microbes by other microbes that inhabit the same environment. The mechanisms employed by some microbes, such as the lactic acid–producing bacteria, in interference are easy to understand. However, the mechanisms used by other types of microbes are not clearly understood.

There are certain criteria that must be met for microbial interference to occur. First, the microorganisms that are producing the inhibiting effect must outnumber the target microorganism. Also, these microorganisms are not usually one specific species, but rather a mixture of many different types of microorganisms. There have been numerous descriptions of the mechanisms that produce the inhibition. Among these are competition for nutrients from the growth substrate, unfavorable changes made to the environment (such as production of acid), and competition for attachment sites. It is most likely that general microbial interference is a combination of these.

Prebiotics, Probiotics, Bacteriophages, and Bacteriocins
The use of individual or varying combinations of prebiotics, probiotics, bacteriophages, and bacteriocins has increased in the past few years. They are included here because of their interrelation to exclusion and interference. While there are many types of each of these currently available, only the general definitions of each type will be given here:

Bacteriophages	Bacteriophages are viruses that are introduced into the environment that eliminate other microorganisms.
Bacteriocins	Bacteriocins are antimicrobial proteins that are produced by other bacteria.
Probiotics	Probiotics are bacteria or other microbes that are introduced into the environment that provide health benefits to the host.
Prebiotics	Prebiotics are carbohydrates that are indigestible by the host but provide nutrients and promote the growth of probiotic bacteria.

Physical Food Protection and Preservation

Other than chemical protection and preservation, there are physical methods of food protection and preservation that can be employed by the processor. These methods vary widely in efficacy against certain microorganisms and may not be suitable for all types of meat production. Physical methods used include modified atmospheres, irradiation, and other processes that employ the electromagnetic spectrum, protection with high temperatures, low temperatures, and low moisture.

Modified Atmospheres

Modified atmospheres are generally used in packaging of the meat product after production. Most modified atmospheres have traditionally been used for the retardation of growth by spoilage organisms, or the extension of shelf life of products. While modified atmospheres will accomplish this goal, many pathogenic microorganisms exhibit sensitivity to these atmospheres as well. Most modified atmospheres involve an increase in the level of carbon dioxide (CO_2) within the packaging.

General Effects of Carbon Dioxide on Microorganisms
Following are some general observations of the use of carbon dioxide (remember that these are general rules and may not always apply, depending on the type of meat product that is being produced):

1. Inhibition of microbial growth by the use of carbon dioxide increases as pH decreases. This is due, in part, to the affect of low pH on microbial growth.
2. The lag phase of growth (refer to Fig. 14.1) is lengthened.
3. The log phase of growth (refer to Fig. 14.1) is reduced.
4. Inhibition of microbial growth by the use of carbon dioxide increases as temperature of storage decreases. This is due, in part, to less than optimal growth temperature for most organisms.
5. Concentrations of carbon dioxide in the range of 20–30% are optimal for microbial inhibition, especially in fresh meats (Gill and Tan, 1980; Gill, 1983).
6. Gram-negative bacteria tend to be more susceptible to carbon dioxide than Gram-positive bacteria. This may present a problem in some foods, as the use of carbon dioxide may cause a

shift in the microbial population to Gram-positive pathogens such as *L. monocytogenes* and the *Clostridia*.

7. Carbon dioxide exhibits an increase in antimicrobial activity when applied under pressure.

Modified Atmosphere Packaging

Modified atmosphere packaging, or MAP, is accomplished by changing the atmosphere within the food package to a composition of gases that is different from normal air. This process is considered hyperbaric, meaning that the pressures involved are higher than normal atmospheric pressure. The typical gases that are used in this process are carbon dioxide, nitrogen, and oxygen. After sealing, the atmosphere within the package cannot be deliberately changed without repackaging. Most packages used for this type of operation are not able to be hermetically sealed, thus the atmosphere within the package may change over time due to the exchange of gases through the packaging material. Currently, there are two types of MAP techniques that are used.

High-oxygen MAP consists of flushing the package with up to 70% oxygen along with varying concentrations of carbon dioxide (20–30%) and/or nitrogen (up to 20%). This method is typical for packaging red meats since the higher levels of oxygen will help to maintain the color of the meat. The levels of carbon dioxide will slow down the growth of aerobic microorganisms, but will not cause cell death in most cases.

Low-oxygen MAP can be used for other meats that do not require the oxymyoglobin to be present for consumer appeal. In these cases, oxygen levels can range up to 10%, while carbon dioxide levels will be in the same range as high-oxygen MAP (20–30%). The remainder of the atmosphere is composed of nitrogen. In the case of low-oxygen MAP, the growth of most aerobic microorganisms is retarded to a significant degree and some cell death may occur.

Vacuum Packaging

Vacuum packaging consists of removing the excess atmosphere from around the product and subsequent sealing in a gas-impermeable package. Some oxygen is removed during the process. Carbon dioxide is typically not added to vacuum-packaged foods. The buildup of carbon dioxide and subsequent decrease in oxygen within the package occurs because of continued cellular respiration by the meat cells and by microbial respiration. Use of vacuum packaging has increased in recent years due to the extension of shelf life that this method exhibits. Vacuum packaging also decreases yield losses and helps to inhibit fatty acid and lipid oxidation. This is an important quality characteristic in meats that contain a high level of unsaturated fats.

Controlled Atmospheres

Controlled atmospheres are not typically used for meat production. In the cases where controlled atmospheres are used, the composition of the gases within the container does not change over time. This is in contrast to MAP, where gas concentrations are expected to change over time. The main reason why controlled atmospheres have limited use in meats is because of the type of packaging that is required. To control the atmosphere and to ensure that no gas exchange takes place across the package, containers made of metal, glass, or laminates of aluminum foil are required. This usually adds too much expense for large commercial operations to use this method. Small specialty operations may use controlled atmosphere packaging for niche markets.

Radiation and the Electromagnetic Spectrum

The *American Heritage® Dictionary* of the English language, 4th edition (2000), defines radiation as "energy radiated or transmitted as rays, waves" or "in the form of particles." The effects of radiation on cells, both eukaryotic and prokaryotic, have been known for more than a century. However, it was not until the latter half of the 20th century that this technique was given serious consideration as a tool for food safety. The use of the electromagnetic spectrum has not reached its full potential for use in foods, mainly because of issues with consumer acceptance. This section will detail the differing types of radiation that may be employed by the food processor in regards to food safety.

Before beginning the discussion of the various radiative methods, it is important to have some definitions of the terms that are used:

The unit for absorbed dose is the Gray (Gy). This is a measure of the energy that is absorbed by the product subjected to radiation. One Gray is equal to 11 joules per kilogram. Most types of waves used in a radiative process for foods have a wavelength of 2000 Angstroms (Å) or less. One Angstrom is equivalent to one-ten millionth of one millimeter or one-two hundred fifty millionth of one inch. The use of radiation for antibacterial purposes is carried out without raising the temperature of the product. This is why the use of radiation is sometimes referred to as cold sterilization or cold pasteurization.

There are also terms that describe the equivalency of a radiative dose to other common antimicrobial practices. *Radurization* is a process that is typically equivalent to low-grade pasteurization. This process substantially reduces the number of viable spoilage organisms on a product to increase shelf life. This process typically involves radiation doses in the range of 0.75–2.5 kiloGray (kGy). *Radicidation* is also preferred to pasteurization. However, in this process the number of viable nonspore-forming pathogens is measured before and after the radiation process. Radicidation will reduce the number of these specific organisms so that none are detectable by standard methods. The typical dose for this process ranges from 2.5 to 10 kGy. Finally, there is *radappertization*. This is a sterilization process that effectively eliminates all microorganisms, including viruses, from the product being treated. The typical dose for radappertization is 30–40 kGy. It should be noted here that radurization and radicidation are the processes that are commonly used on fresh meats, with radurization being more common. This is because of the undesirable quality aspects that occur with high doses of radiative energy. Radappertization is used in the canning industry.

The two most common ways that radiation is applied to meat products before sale to the consumer are the use of gamma rays and the use of electron beams. These two processes are discussed below.

Gamma Rays or Gamma Radiation

Gamma rays for food irradiation are the products of either cobalt-60 (^{60}Co) or cesium-137 (^{137}Cs). These elements are relatively inexpensive and are the stable by-products of atomic fission. In most cases, the radioactive cobalt or cesium is placed on an elevator within a concrete and lead enclosure. The enclosure has a pool of water at the bottom. When not in use, the radioactive source is lowered into the pool of water so that products can be placed within the chamber or if maintenance needs to be performed. Housing the material in the water when not in use allows personnel to safely enter the chamber. When the source is needed for irradiation of products, the products are placed around the radioactive source at specific distances for desired doses, personnel are removed from the chamber, and the source is raised. After the specified time has passed for the dose to be received by the product, the source is lowered back into the pool of water and the product can be removed from the chamber. Use of gamma rays for antimicrobial activity does not impart radioactivity to the product. The gamma rays pass through the product and are absorbed by the concrete and lead in

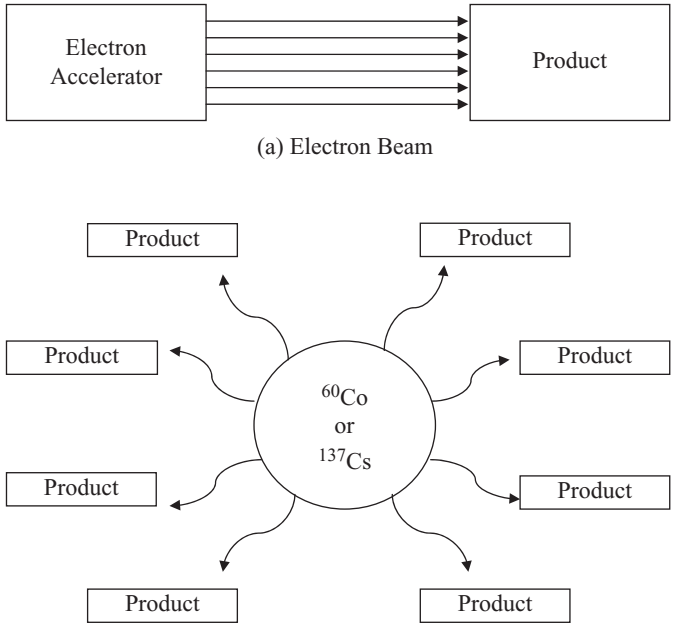

Figure 14.3 Diagrammatic representation of electron beams (a); and gamma rays (b) for use in food processing.

the chamber. There are disadvantages to using either radioactive cobalt or cesium for antimicrobial results on meats. One of the main issues is that the source of the radiation cannot be switched on or off immediately. Second, the cost of construction of the housing for the procedure may be expensive. Finally, the half-life of cobalt-60 is just 5 years. This means that the radioactive source might have to be replaced many times during the life of an operation. Disposal of this element may also be expensive. The alternative to this issue is the use of cesium-137, which has a half-life of approximately 30 years (Fig. 14.3).

Electron Beams

Electron beams are focused streams of electrons that are generated from an electron accelerator. Electron beam energy as it is associated with food preservation is measured in Grays, just as gamma radiation. Other than the different sources of electromagnetic energy, there are also differences in gamma radiation and electron beams that may entice a producer to use one method over the other. In general, the ease of penetration into the irradiated product is easier with gamma rays rather than electron beams. However, the energy emission from an electron accelerator can be adjusted so that penetration of thicker products by the electron beams can be accomplished. There is also the issue of emission rate or dosage capacity. In a gamma-ray emitter powered by cobalt-60, the emission rate for the gamma rays is between 1 and 100 Grays per minute. Today's electron accelerators can emit electrons toward a product at 1000 to 1,000,000 Grays per second. Other advantages of electron beams over gamma radiation were described by Koch and Eisenhower (1965) and include:

1. The ease of variability of the electron beam current and energy imparts flexibility in the choice of surface and depth treatments for food items.

2. The setup of an electron generator to emit the radiation in one direction only allows for flexibility in machinery and package design.
3. Electron generators can be easily switched off and on. This gives the producer the ability to shut the machinery down if it is not needed without any need for continued maintenance and it allows the radiation source (electron accelerator) the ability to be transported without the use of a radiation shield.
4. Electron accelerators have the ability to be programmed and regulated easily. This allows many different products, including those with small, intricate, or non-uniform shape to be irradiated efficiently.

Ultraviolet Light

Although ultraviolet (UV) light is not considered ionizing radiation, it is included here because it is part of the electromagnetic spectrum. UV light is highly bactericidal because of the resulting mutations in the nucleic acids of the target cells. Also, unlike gamma rays and electron beams, the most effective wavelength of UV light to produce antimicrobial effects is greater than 2000 Å, at approximately 2600 Å. Although UV light is highly bactericidal, there are problems with its use in the meat industry. UV light does not penetrate very deeply into target substances, so surface decontamination is the main option for its use. UV light may also cause rancidity in fatty acids and lipids or cause product discoloration.

Microwave Energy

Like UV light, microwave energy is not an ionizing radiation, but it is part of the electromagnetic spectrum. Microwave energy acts on products by producing heat from friction between molecules that are oscillating because of changes in the electromagnetic field that surrounds them. The production of heat is the mechanism by which microwave energy is inhibitory or the cause of cell death. This makes microwave energy unusable for ready-to-cook products. However, microwave energy is used primarily by consumers for reconstitution of partially cooked or frozen products. Another issue with the use of microwave energy is unevenness in cooking. Most of the research that has been carried out with the use of microwave energy in foods has been done at one of two frequencies of microwaves, either 915 or 2450 megacycles. At 915 megacycles, the molecules that are oscillating move back and forth 915 million times per second (Goldblith, 1966). Molecules move at two billion four hundred fifty million times per second for 2450 megacycles.

High-Temperature Protection

High-temperature protection of foods from microbial activity stems from the destructive effect of heat on microbes. For use in this context, high temperature refers to any temperature above ambient temperature of the product when subjected to the treatment. For most foods, there are two types of high-temperature protection: pasteurization by heat, and sterilization by heat. The pasteurization concept refers to one of two different results: (1) the destruction or reduction of spoilage organisms in the product or (2) the destruction of all pathogenic organisms within the product. Sterilization by high temperatures refers to the destruction of all microorganisms in a sample so that none are recoverable by standard methods.

All bacteria possess a certain degree of heat tolerance, but this tolerance varies widely between organisms. The presence of other components within the medium that is subjected to high temperatures will also affect the heat tolerance of microorganisms, either by increasing the tolerance or lowering it. These include:

1. Initial microbial load.
2. Water activity.
3. pH.
4. Carbohydrate concentration.
5. Protein concentration.
6. Fat concentration.
7. Salt concentration.
8. Microbial age.
9. Presence of other microbial inhibitors.

Heat Destruction and Thermal Death Time

Since heat tolerance varies widely among microorganisms, a calculation is necessary to determine the requirements that must be met in order to kill or significantly inhibit the target organism(s). Thermal death time, or TDT, is the time that is required to kill a given number of target microorganisms at a given temperature. In this context, a specific number of microbes are placed within a medium and subjected to heat treatment at a certain temperature. The time that it takes to kill all of the microorganisms is referred to as the TDT.

D Value

Another important concept when using heat to destroy microbes is *D* value, or decimal reduction time. The *D* value of an organism is the time required to kill 90% of a known population of microbes at a given temperature. This means that for a given temperature there is a 1 $\log_{10}$ reduction in the number of microorganisms. For example, if the $D_{250^\circ F}$ for an organism is 2 min, a sample containing this organism at an initial load of 10^6 microorganisms would be expected to have 10^5 microorganisms after a 2 min heat treatment at 250°F. After 10 min, the detectable amount would be 10 microorganisms. Depending on the type of organism that is being tested and the temperature applied, *D* values can range from a few seconds to minutes and hours. Many *D* values have been calculated for the temperature of 250°F. Remember that when expressing *D* values, a time unit should always accompany the number. An example of a thermal death curve and the calculation of *D* value is shown in Figure 14.4.

z Value

While it is important to know the *D* value for a given microorganism at a specific temperature, there is more information that is needed to determine a thermal process for destroying that organism on a meat or food product. With the use of *D* value and the inclusion of *z* value, this is possible. The *z* value for an organism is a measure of the degrees (in Fahrenheit) that is required for the *D* value to be reduced by 90% or 1 $\log_{10}$. While *D* value gives the investigator information on the heat tolerance at a given temperature, the *z* value gives the investigator the resistance to destruction at differing temperatures. It is important to note here that calculation of the *z* value for a microbe depends on knowing at least two *D* values for that microbe. An example of the use of *z* values in the determination of thermal destruction process follows. If a thermal process for the destruction of an organism is considered valid when the temperature is 150°F for 5 min and the *z* value for

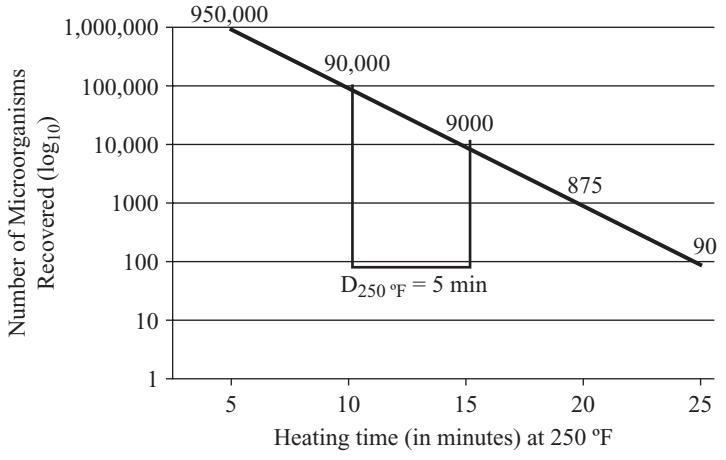

Figure 14.4 Diagrammatic representation of a thermal death curve and the calculation of *D* value.

the organism in question is 10, then an equivalent destruction of that organism will happen in 50 min if the temperature is lowered to 140°F. If the temperature is raised to 160°F, the time that it takes to achieve an equivalent thermal destruction will decrease to 0.5 min, or 30 s. A diagrammatic representation of the above example is shown in Figure 14.5.

Safe Harbor Lethality and the 12D Concept

Lethality for microorganisms by heat treatment is different for different types of foods. Meats that are not placed in a final package that requires a retort process (i.e., canning) do not have the same lethality requirements as canned products. For non-retort packaged meats, the heat treatment should be designed and validated such that the elimination of pathogenic vegetative cells is achieved. This

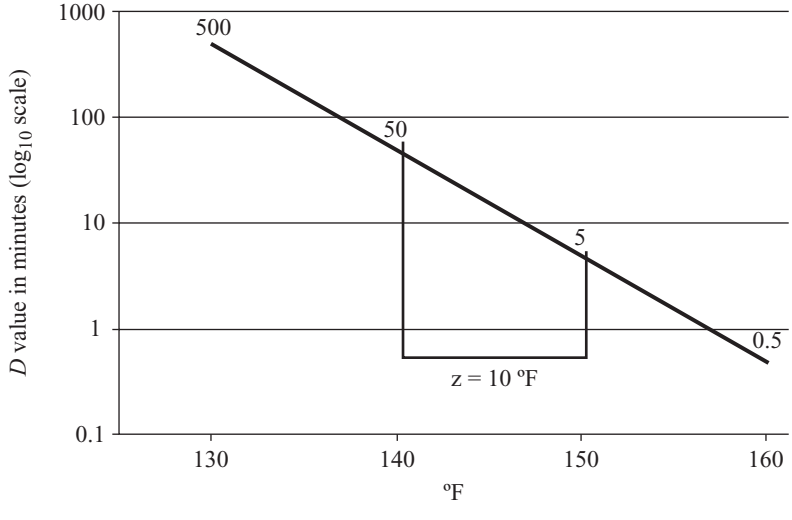

Figure 14.5 Diagrammatic representation of a thermal death curve and the calculation of *z* value (150°F for 5 min is assumed to be an adequate process).

Table 14.6 Common safe harbor lethality temperatures and times for common meat products

Product	Time	Temperature
Poultry	15 s	165°F (73.9°C)
Stuffed meats, stuffed poultry	15 s	165°F (73.9°C)
Ground meats (beef, pork, poultry)	15 s	155°F (68.3°C)
Whole muscle beef	No time limit	140°F (60°C)
Whole muscle lamb	No time limit	140°F (60°C)
Seafood	No time limit	140°F (60°C)
Preprocessed foods	15 s	145°F (62.8°C)

is referred to as safe harbor lethality. Safe harbor lethality processes will reduce the viable count of *Salmonella* serotypes and other pathogens commonly found on meats by 5 to 7 $\log_{10}$(s). It is commonly accepted that once a meat has reached an internal temperature of 160°F (71.1°C), this will provide safe harbor lethality against nonspore-forming bacteria such as *S. aureus*, *Campylobacter jejuni*, *L. Monocytogenes*, and many *Salmonella* serotytpes (FSIS, 1999a) (Table 14.6).

While safe harbor lethality is effective against nonspore-formers at the heat treatment site, there are pathogenic organisms that produce spores, and recontamination of products by nonspore-forming pathogens can occur. One such organism that produces spores and is important to meat producers is *C. perfringens*. For these spore-formers and to protect against outgrowth by recontamination, processors are required by USDA to cool their products quickly after heat treatment. This process is referred to as product stabilization. USDA product stabilization performance standards ensure that spores of *C. perfringens* will not germinate to viable cells (FSIS, 1999b). In general, the guidelines for stabilization require that during post-cook cooling, products should be cooled in such a manner that the product temperature does not remain between 130°F (54.4°C) and 80°F (26.6°C) for more than 1.5 h. Products should also not remain between 80°F (26.6°C) and 40°F (4.4°C) for more than 5 h. The final product temperature should be at or below 40°F (4.4°C).

For those products that undergo a retort process during production, a 12 $\log_{10}$ or 12-*D* process is required. Canned products are very susceptible to outgrowth of spores of *C. botulinum* because of the anaerobic atmosphere of the package. *C. botulinum* produces a toxin that causes the disease botulism. The 12-*D* process will reduce the probability of survival of the most heat-resistant *C. botulinum* spores to 1 in one trillion (10^{12}) containers. The 12-*D* process is only used for products that have a final pH above 4.6, since *C. botulinum* spores will not germinate below this pH.

Low-Temperature Protection

Low-temperature protection of foods is based on the effect of low temperatures on most microorganisms. Most microbial growth can be reduced by decreasing temperatures to the freezing point of water. Many microbes will essentially stop growth and reproductive functions at temperatures below the freezing point of water. This is because metabolic reactions within microbes are due to the action of enzymes within the cell. Enzymatic reactions decrease in low temperatures. In general, there is a 1.5–2.5 increase in the rate of enzymatic reactions for each increase in temperature of 10°C. The inverse is also true. This clearly shows how microbial growth and reproduction can be decreased by decreasing the temperature of the meat during production and storage.

Most meats are routinely chilled via immersion in cold water or via cold ambient air temperature to 40°F (4.4°C) or below before packaging. Some meats are kept at this refrigeration temperature

Table 14.7 Differences in slow freezing and fast freezing

Slow freezing	Fast freezing
Formation of large ice crystals	Formation of small ice crystals
Gradual adaptation by microbes to temperature	No adaptation by microbes to low temperatures
No thermal shock effect to microbes	Thermal shock effect to microbes
Accrual of concentrated solutes within the cell has beneficial effects	No solute accrual (no beneficial effects)

during transportation, storage, and when presented to the consumer for sale. However, many meats are frozen to help extend shelf life or for sale to the consumer as a frozen product. The freezing of meat products is done by either fast freezing or slow freezing. Fast freezing is said to occur when the product temperature is lowered to -4°F (-20°C) or below in 30 min or less. Slow freezing happens when the product is lowered to the desired freezing temperature before 72 h has elapsed. The differences in slow freezing versus fast freezing are illustrated in Table 14.7.

With respect to quality of the meat product that undergoes freezing, fast freezing is more advantageous than slow freezing. This is mainly because of the differences in ice crystal formation. Since fast freezing does not allow for the formation of large ice crystals within the meat, there is less damage to the meat via cell membrane, cell wall, and internal structure disruption. In addition, foods that undergo a slow freezing process tend to lose more water in the form of drip loss than those products that are frozen in a fast process.

Thawing

Thawing of a product is an inherent part of the freezing process. Repetitive freezing and thawing of a meat product disrupts the cell membranes of microorganisms causing cell death. However, this process is also detrimental to the quality of the meat product. Thawing typically takes longer than freezing when performed over the same temperature differentials. This can pose problems for meat products that are contaminated with psychrophilic organisms, as it affords the microbes the opportunity to grow and reproduce.

Low-Moisture Protection

Water available for biological processes is essential for the growth and reproduction of microorganisms on meat and food products. The amount of the total water available for these biological processes is referred to as water activity (a_w) and is expressed as a decimal percentage between 0.00 and 1.00. Refer to Table 14.2 for the minimum a_w of microorganisms commonly associated with food and meat products. Reduction of a_w levels to below 0.60 and total moisture to below 25% are the conditions that must be met before a food can be considered a dried or low-moisture food. Meats are typically cooked before drying. The final total moisture content for meats after the drying process has been completed should be around 4%.

Microbial Indicators and Sampling Plans

Certain microorganisms are used as indicators of safety and quality in meat and food products. Most indicator organisms are used for food safety purposes or to assess the effect of sanitation procedures. Organisms used to predict quality typically refer to shelf life predictions.

Product Quality Prediction by Microbial Numbers

Generally, microbial indicators of product quality are product specific, since varying growth conditions exist on different products. However, there are certain criteria that should be looked for when determining an indicator organism. These include but are not limited to the following: (1) the growth and numbers of the organism should be inversely correlated with the quality of the product; (2) other microorganisms that are known to be in the natural microflora of the product should not negatively affect the growth of the indicator organism; (3) detection and/or enumeration of the indicator organism should be easily accomplished in a short period of time; and (4) the organism should be detectable in the food which is being assessed for quality.

Along with direct enumeration of indicator organisms, quality of a product can also be determined by the presence and quantity of metabolic products of microorganisms that are associated with spoilage. Examples of these chemicals are cadaverine and putrecine in vacuum-packaged beef.

Product Safety Prediction by Microbial Numbers

Just as with quality prediction by indicator organisms, there are certain criteria that must be met when determining a food safety indicator organism to indicate the presence of a particular pathogen in a meat or food product. These include (1) the indicator organism should be easily discernible from other microorganisms that may be present on the product; (2) the indicator organism should always be present when the pathogen is present in the product; (3) the indicator organism should be detected and/or enumerated easily in a short period of time; (4) the indicator should express a growth curve that is higher during all phases than the pathogen (Fig. 14.6); and (5) the indicator should not be present in products that are free of the pathogen except in minimal numbers.

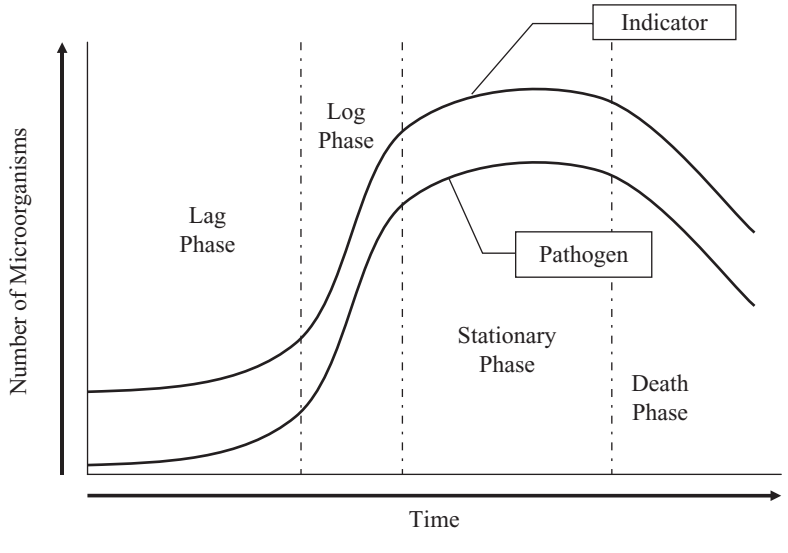

Figure 14.6 Ideal growth curve relationship of pathogen and indicator organism.

Whether or not an indicator organism is selected for quality or safety purposes, some other issues must be taken into account. In many situations, enumeration of coliform bacteria has been used to indicate process control of sanitation and other processing methods as these organisms have been linked to enteric pathogens. However, the presence of psychrotrophic members of the family Enterobacteriaceae can inflate normal coliform count numbers to unacceptable levels when there may not actually be a problem with process control. Indicator organisms may also be used to determine process control in situations where the presence of the pathogen can result in serious consumer injury and product recalls. An example of this would be using *Listeria* spp. or *Listeria*-like organisms to test for the presence of *L. monocytogenes*. In this instance, the presence of the indicator can signify that further testing on a sample or lot needs to be done before shipment.

Microbial Sampling Plans

Microbiological sampling plans are used throughout the meat production industry. This section provides an overview of microbiological sampling plans that will enable the reader to be able to understand the concepts. However, before implementing or recommending a formal plan, producers should consult a food safety expert and/or one of the most comprehensive texts on microbiological sampling available: *Microorganisms in Foods 7: Microbiological Testing in Food Safety Management* by ICMSF, ©2002.

A microbial sampling plan is used to determine the acceptance or rejection of a "lot" of product based on the results of microbiological testing. Lot sizes can vary widely in size, but the basic sampling plan will remain the same. There are two types of plans that can be used. The most common type of plan is the attribute plan. This type of plan bases the acceptance or rejection on attributes of the samples, that is, presence or absence of a particular organism or the levels of an organism. The other type of plan is the variable plan. These plans are used when the frequency distribution of a particular microorganism is known. Variable plans are much more intricate and can prove more useful in certain circumstances than attribute plans, but most food and meat processors use attribute plans.

Attribute Sampling Plans

There are two types of attribute sampling plans that are employed in the meat industry today: the two-class attribute plan, and the three-class attribute plan. Each plan bases the acceptance or rejection of a lot based on the results from a required number of samples performed by specific standard methods.

Two-class attribute plans are the simplest form of sampling plan that can be used. For this plan, there are certain measurements that must be made before the decision to accept or to reject is made. Definitions of these measurements can be found below:

n: The number of units from the lot that must be subjected to sampling.
c : The upper limit of samples that may surpass the microbiological condition set out in *m*. When *c* is exceeded, the lot is rejected.
m: The upper limit of key bacteria per gram of sample.

Many two-class attribute plans are presence/absence (qualitative) sampling plans where *m* is assigned a value of zero. An example of this type of plan would be *E. coli* presence in beef. If *n* for the lot is 10 and the *c* is zero, then 10 samples must be taken from the lot and all 10 of these samples must

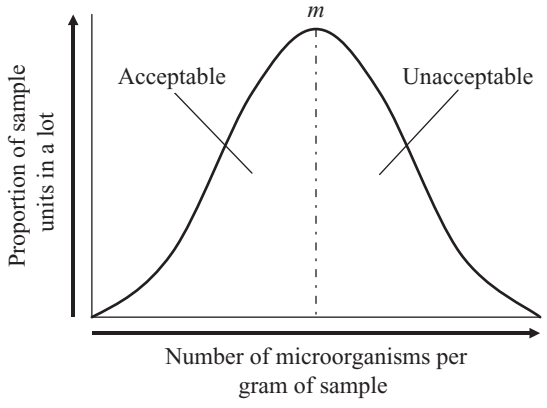

Figure 14.7 Graphical representation of a two-class attribute sampling plan.

be negative for the presence of *E. coli* for the lot to be accepted. In other cases, if c has a value greater than zero, then the number of samples positive for the presence of the organism must be at or below c for the lot to be accepted. A graphical representation of a two-class attribute sampling plan is given in Figure 14.7.

Three-class attribute plans also use the measurements of n, c, and m, but they also have another measurement, M. This measurement, M, is set to a level that separates marginally acceptable product from unacceptable product. In the three-class attribute plan, m is not set to zero as is the case with many two-class attribute plans. Three-class plans are quantitative. An example for this type plan would be one where the aerobic plant count (APC) of a certain food was measured. In this sample case, $n = 10$, $c = 3$, $m = 10^2$, and $M = 10^4$. For this case, if no more than 3 samples of the 10 return a result over 10^2, the lot is considered acceptable. However, if more than 3 samples of the 10 return a result over 10^2 or if any sample of the 10 returns a result over 10^4, then the lot is rejected. A graphical representation of a three-class attribute sampling plan is given in Figure 14.8.

When deciding whether to implement a two-class or three-class sampling plan to a particular product, a few questions must be answered. If no positive samples can occur in a product, then

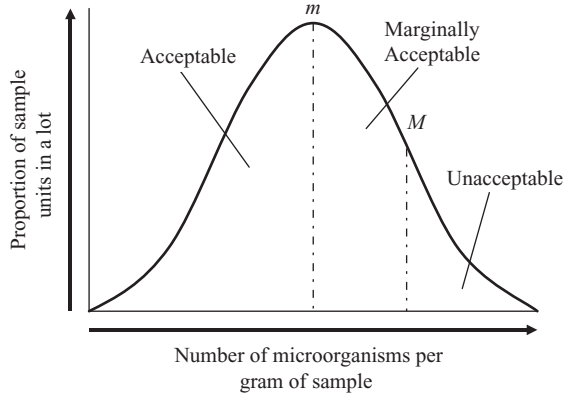

Figure 14.8 Graphical representation of a three-class attribute sampling plan.

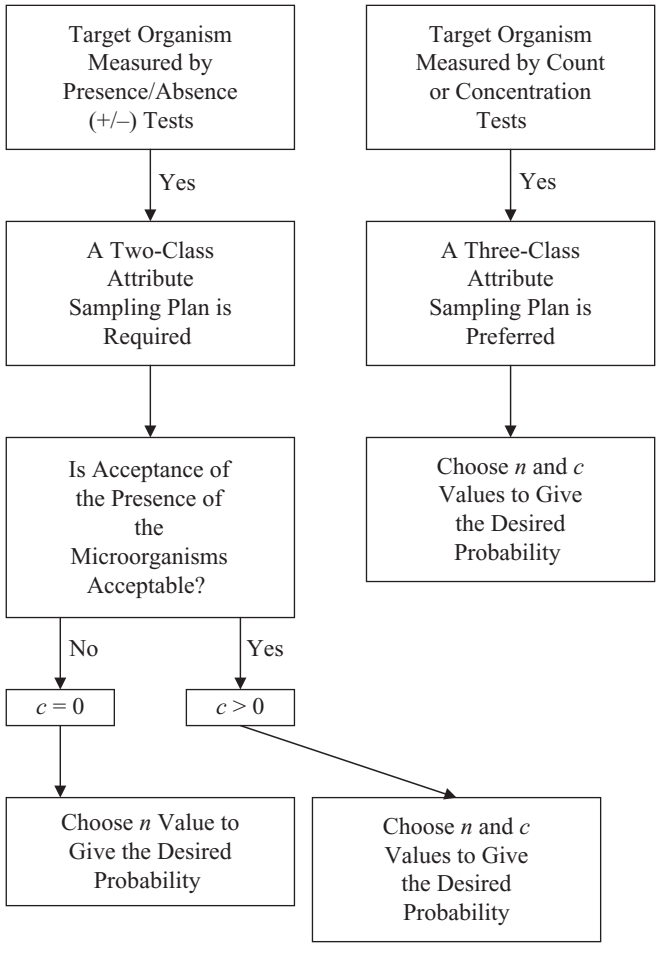

Figure 14.9 Choosing between a two-class and a three-class attribute sampling plan.

a two-class sampling plan would be appropriate with c valued at zero. A flow-chart for deciding between a two-class and three-class attribute sampling plan is given in Figure 14.9.

Implementation of attribute plans is based on severity of the type of hazard that can be identified by use of the sampling plan and the use of the meat or food product. The International Commission on Microbiological Specifications for Foods (ICMSF) has identified 15 "cases" where attribute sampling plans can be used. These "cases" assess the degree of concern relative to the food safety hazard (see below) and the conditions in which the food is expected to be handled and consumed after sampling for the attribute class plan has occurred. The conditions in which the food is expected to be handled are classified into three categories: (1) reduction in the degree of hazard; (2) no change in the degree of hazard; and (3) possible increase in the degree of hazard. The degree of concern is classified into five categories which are outlined thus:

1. **Utility**: General contamination, contamination that will result in the reduction of shelf life or spoilage (no direct health hazard).

2. **Indicator**: Low concern for health consequences (indirect health hazard). Applies to situations where indicator organisms are used to assess food safety.
3. **Moderate**: A direct health hazard with limited spread of the hazard expected to occur.
4. **Serious**: A direct health hazard that poses health risks that may be incapacitating, but not usually life-threatening. These hazards typically have a moderate duration and the threat of acute or chronic sequel is rare.
5. **Severe**: A direct health hazard that poses risks for the general population or a restricted population that is expected to consume the food. The risks of the hazard are typically life-threatening, cause illness of a long duration, and may be linked to chronic sequel.

The "cases" proposed by ICMSF range in number from 1 to 15 with the implied severity of the hazard increasing as the "case" number rises. Table 14.8 contains information related to each "case" and the ICMSF recommended sampling plan that should be employed in each instance. Further information regarding "cases" can be found in *Microorganisms in Foods 7: Microbiological Testing in Food Safety Management* by ICMSF, ©2002.

Table 14.8 The 15 "Cases," as proposed by ICMSF of attribute sampling plans

Case 1 • Utility hazard • Possible reduction in spoilage hazard (Increase in shelf life) • Three-class plan recommended $n = 5$ $c = 3$	**Case 2** • Utility hazard • No change in spoilage hazard • Three-class plan recommended $n = 5$ $c = 2$	**Case 3** • Utility hazard • Possible increase in spoilage hazard (Decrease in shelf life) • Three-class plan recommended $n = 5$ $c = 1$
Case 4 • Indicator hazard • Possible reduction in hazard • Three-class plan recommended $n = 5$ $c = 3$	**Case 5** • Indicator hazard • No change in hazard • Three-class plan recommended $n = 5$ $c = 2$	**Case 6** • Indicator hazard • Possible increase in hazard • Three-class plan recommended $n = 5$ $c = 1$
Case 7 • Moderate hazard • Possible reduction in hazard • Three-class plan recommended $n = 5$ $c = 2$	**Case 8** • Moderate hazard • No change in hazard • Three-class plan recommended $n = 5$ $c = 1$	**Case 9** • Moderate hazard • Possible increase in hazard • Three-class plan recommended $n = 10$ $c = 1$
Case 10 • Serious hazard • Possible reduction in hazard • Two-class plan recommended $n = 5$ $c = 0$	**Case 11** • Serious hazard • No change in hazard • Two-class plan recommended $n = 10$ $c = 0$	**Case 12** • Serious hazard • Possible increase in hazard • Two-class plan recommended $n = 20$ $c = 0$
Case 13 • Severe hazard • Possible reduction in hazard • Two-class plan recommended $n = 15$ $c = 0$	**Case 14** • Severe hazard • No change in hazard • Two-class plan recommended $n = 30$ $c = 0$	**Case 15** • Severe hazard • Possible increase in hazard • Two-class plan recommended $n = 60$ $c = 0$

Source: Adapted from *Microorganisms in Foods 7*, ICMSF, ©2002.

Microbiological Sampling of the Environment

Direct microbiological sampling of products is an easy way to determine the acceptance or rejection of a lot of product. However, in many instances, microbiological sampling of the environment will provide the processor with information that includes possible points of initial contamination and possible points of cross-contamination and re-contamination. This information can be used to strengthen the GMPs and SSOPs that are used in the plant. Strengthening these prerequisite programs will also strengthen the effectiveness of the overall FSOs or HACCP plan. Even though a food processor may exert adequate control over CCPs to ensure the destruction of pathogens on raw materials entering the processing plant, there are cases where the product is still contaminated with a microbe of concern. In these cases, the most likely contributor to the contamination is either (1) addition of a contaminated ingredient following the raw product kill step or (2) cross-contamination from the environment (ICMSF, 2002). For the instances where ingredients are added after the kill step, microbiological sampling of the ingredients will determine if the ingredient in question should be accepted for use or rejected. Sampling plans for ingredients can be set up using either a two-class or three-class attribute system, just as with lot sampling.

Sampling of the environment is a bit more intensive, but no less important. Typical environmental sampling plans are used to accomplish the following tasks: (1) determine the risk of contamination that the environment poses to the product; (2) establishment of a baseline account of the presence and numbers of microorganisms found in the processing environment so that a comparison with subsequent sampling will determine if the processing area is considered to be under control; (3) assessment of the overall production environment to determine if corrective actions should be taken.

Most environmental sampling centers on a set of microorganisms such as the APC, a specific pathogen such as *L. monocytogenes* or a specific indicator organism such as *E. coli* biotype I. Microbiological data accumulated during the baseline sampling period will help the processor to determine the locations where subsequent sampling should occur, what types of samples should be taken, and the regularity of sampling. Many processors will divide the processing facility into sectors or zones for environmental sampling. These zones may or may not be sampled for the same microbes depending on the baseline data. Sampling frequency may also vary between the zones. Processors may have different names for the zones in their facility, but they are typically grouped into categories that range in risk from high to low. Areas in the processing facility that are grouped into the highest risk category for possible contamination include all product contact surfaces. These areas will have the greatest sampling frequency. Surfaces that do not come into contact with the product but are in close enough proximity to the product where contamination may occur include the exterior surfaces of some equipment and the floors of the processing area. These surfaces should be sampled frequently but not as frequently as product contact surfaces. Surfaces such as hallway walls and floors, office telephones, and desk chairs should still be tested, but only periodically (once or twice per year). Sampling schedules for the environment can be set up using either the two-class or three-class attribute system. These plans are typically set up so that corrective actions are taken and an increased sampling frequency occurs if a deviation occurs.

Common Microbiological Culturing Methods

The use of the previous methods of distinction between “cases” and the concept of microbiological quality and safety are of no use unless the investigator or personnel responsible for determining lot safety can accurately determine the presence and/or number of microorganisms in a sample. For this reason, there are many methods that are currently available for the determination of microbiological

safety and quality. Many microorganisms have specific procedures that must be followed for their culture. However, many of these methods directly relate back to one of four techniques that have been used repeatedly for isolation and qualitative analysis. These methods are (1) most probable number method; (2) plate count method; (3) direct microscopic counts; and (4) dye reduction methods.

Most Probable Number Method

The most probable number, or MPN, method is widely used to determine microbial numbers in a sample. There are two types of this method that can be employed: the three-tube method, or the five-tube method. There are advantages to using this procedure that include (1) simplicity of the procedure; and (2) since MPN is a statistical test, results from multiple MPN procedures on the same sample have less included variability than comparable plate count methods. The major disadvantage of using the MPN method is the considerable use of glassware, especially for multiple samples.

Varying media (selective, differential, or both) can be used for the determination of specific organisms. For this procedure, appropriate dilutions of a representative sample are prepared. After preparation, three serial dilutions are placed into either 9 or 15 tubes for the three-tube method or five-tube method, respectively. After incubation in the MPN tubes at an appropriate temperature and atmospheric conditions (e.g., aerobic), the tubes are examined for growth. The count for the target organism in the original sample is then calculated using standard MPN tables.

Plate Count Method

This method is also one of the most widely used techniques for determining microbial presence and numbers in a sample. Although the media in which the culturing takes place can vary by the target organism, the basic technique remains the same. There are many ways to collect a sample for the plate count method. A representative sample (e.g., 25 g) can be combined with an appropriate volume of a diluent and blended or stomached to ensure homogenization of the sample. Other methods for collecting a sample include swabs or rinses.

Appropriate dilutions are made to ensure that a plate from the sample is in the standard countable range for the medium used. At this point, for some organisms, a process of enrichment may be included. This involves incubating the sample in an appropriate enrichment medium (usually broth) for a certain amount of time to make sure that any cells that may be injured are represented, or to make sure that replication of cells that may be low in numbers in the sample are represented. If a sample is subjected to enrichment, the concluding test is considered to be a presence/absence or qualitative test since the microorganisms are allowed to reproduce while in the enrichment. After the appropriate dilutions are made (or after enrichment), the dilutions are plated onto an agar medium that is suitable for the target organism. The plates are then incubated at an appropriate temperature and atmospheric conditions for the target organism. After incubation, the plates are examined and counted either by hand or by an electronic counter. For all plate count test, a selective or differential agar should be used if one is available or unless the test is for a total count of the organisms present in the sample (e.g., total aerobic count). If a selective or differential medium is not available, biochemical tests should be employed to make sure that the organism recovered is the target organism. Examples of media used for specific microorganisms include brilliant green sulfa agar (BGS) or xylose lactose tergitol 4 (XLT-4) for *Salmonella* spp., eosin methylene blue

agar (EMB) or violet red bile agar (VRB) for enterobacteriaceae, and *E. coli* and modified oxford agar (MOX) for *Listeria* spp.

Direct Microscopic Count Method

Direct microscopic counts consist of preparing slide smears of the sample, staining with a dye, and directly counting the number of cells on the slide. This method is not readily used by the meat industry since the main distinction that can be made is that of cell morphology. Since samples of meat products may contain morphologically similar microorganisms of different spoilage or pathogenic capacity, this method is not practical.

Dye Reduction Method

In the dye reduction method, an estimate of the number of microorganisms in a sample is obtained by the addition of prepared supernatants to dyes. The time that it takes to reduce the dyes is measured and a resultant estimate of the microbial population is obtained, as the time it takes for dye reduction is inversely proportional to the microbial population. For this method, either methylene blue or resazurin is used as the dye. Methylene blue changes from blue to white when it is reduced, while resazurin changes from slate blue to pink or white. The dye reduction method can be used for meats, but the presence of natural reductive substances in raw meat may pose a problem. Cooked meats do not contain the same large amounts of natural reductants as raw meats, therefore this test is applicable for these products. One solution to the presence of reductants in raw meats is to use a stomacher for homogenization of the sample rather than a blender.

Other Microbial Methods

There are other methods of microbial detection and enumeration that are currently available to meat producers. These include enzyme-linked immunosorbent assay (ELISA) and so-called quick tests that typically use biochemical reactions to determine microbial presence or populations. These tests tend to be highly specific for certain microorganisms and will not be covered in this text. Producers are encouraged to explore all options when determining the appropriate type of microbiological test to be used in their operation. Monetary and time constraints, along with test sensitivity, tend to be the major factors in deciding which type of test to use.

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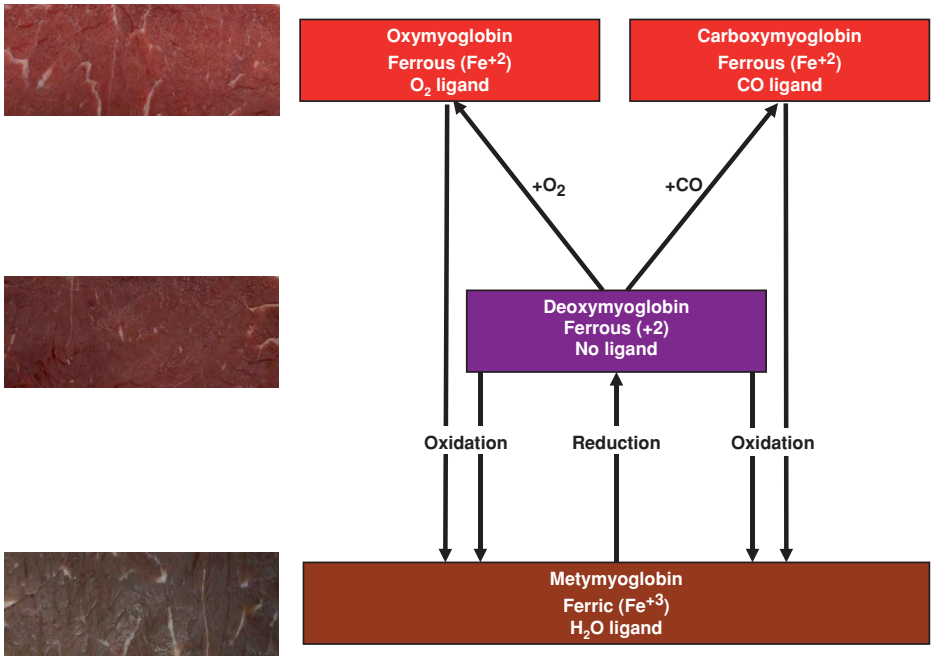

Plate 9.1 The effects of myoglobin redox state, ligands, and iron valence on the surface color of postmortem muscle.

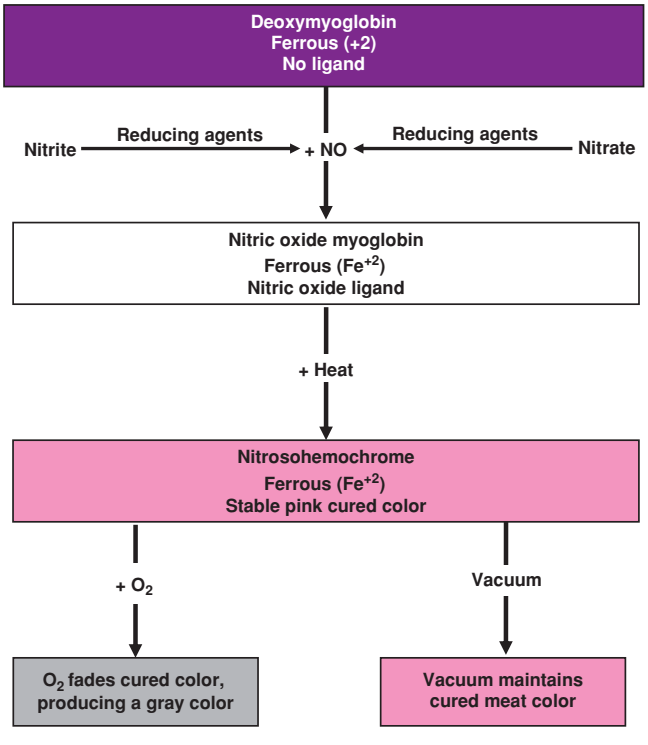

Plate 9.2 Cured color formation and fade.

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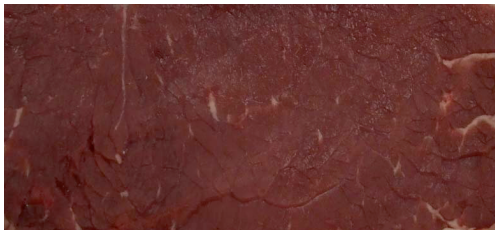
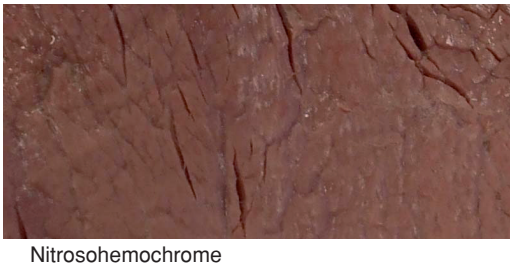


Deoxymyoglobin

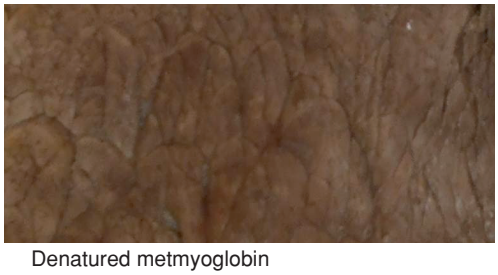
Nitric oxide myoglobin

Oxymyoglobin

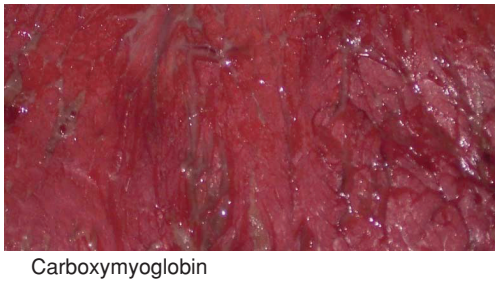


Nitrosohemochrome

Metmyoglobin



Denatured metmyoglobin



Carboxymyoglobin

Plate 9.3 Colors associated with myoglobin forms on the surface of beef.