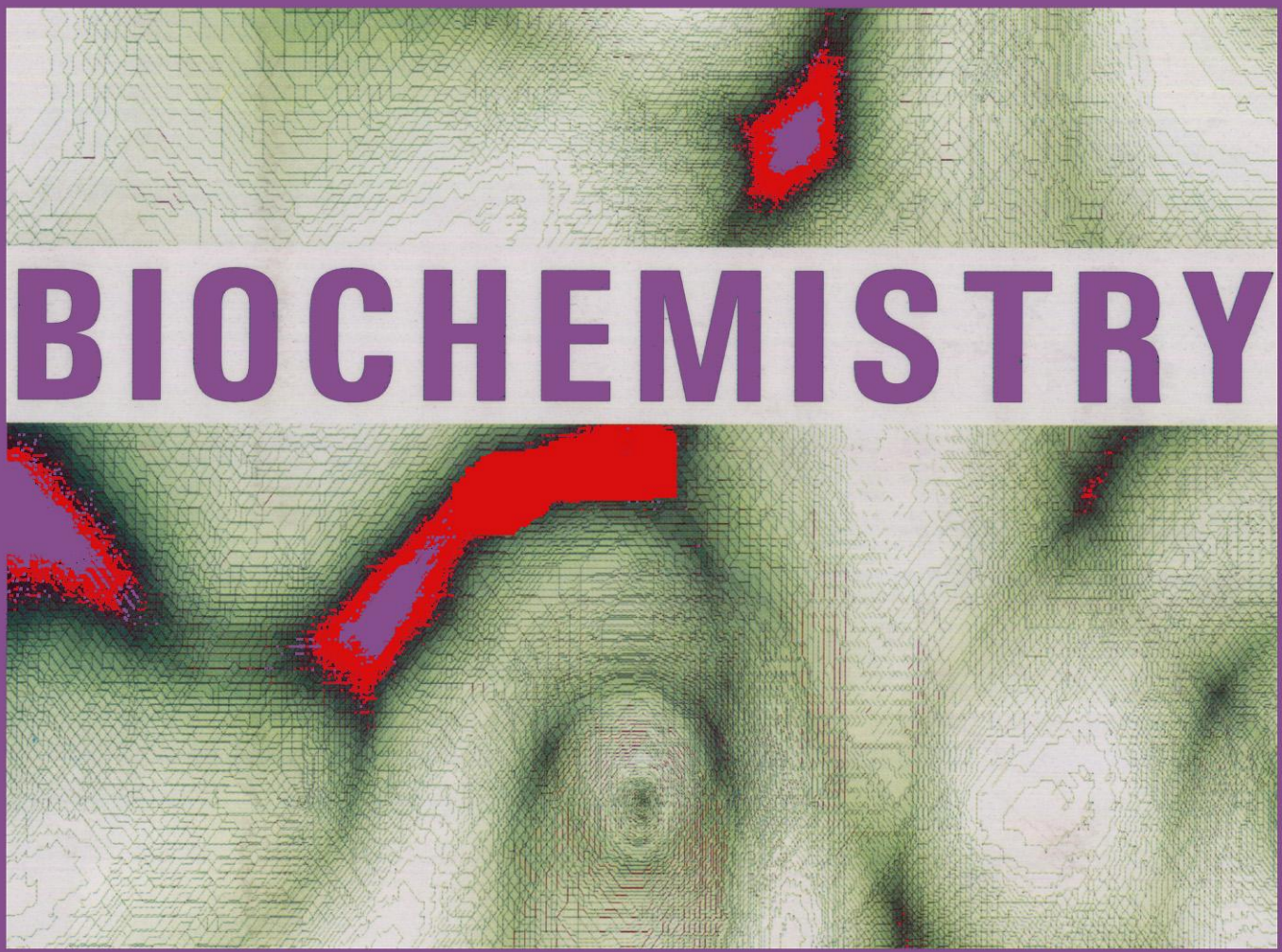

BIOCHEMISTRY

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HIMALAYA PUBLISHING HOUSE

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Dedicated
to
Late Mr. D.P. Pandey
who had nourished HPH with
his hard labour and sincere dedication

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1 Introduction to Biochemistry

1.1 Meaning of Biochemistry

The term *'Biochemistry'* (*Bios*^{G} = life) was first of all introduced by a German chemist called Carl Neuberg in 1903. It may be defined as, the science which describes, in the language of chemistry, the structure and functioning of living organisms.

Biochemistry also means the chemistry of living organisms. Biochemistry includes aspects of Organic Chemistry, Inorganic Chemistry, Physical Chemistry, Physics, Biology and other basic disciplines. It is also interrelated with Physiology, Microbiology, Clinical Sciences and Agriculture.

Biochemistry may also be defined more formally as the science concerned with the chemical basis of life (Gk *bios* 'life'). The cell is the structural unit of living systems. Consideration of this concept leads to a functional definition of biochemistry as the science concerned with the chemical constituents of living cells and with the reactions and processes they undergo. By this definition, biochemistry encompasses wide areas of *cell biology* and all of *molecular biology*.

Biochemistry may be defined as that branch of science which deals with the chemical processes that go on in living matter, ranging from viruses and bacteria to plants and animals. Actually, it is a study of biology at molecular level. It is concerned with the molecules that make up the structures of cells and organs, *i.e.*, molecular anatomy. Much is known about these molecules, and rapid progress is being made in working out the molecular organisation of the cell.

As biochemistry deals with carbon compounds and the reactions they undergo in living organisms, it implies organic chemistry, the chemistry of carbon compounds, is basic to

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biochemistry. The chemistry of natural products and biochemistry overlap extensively and lack a clearly defined border.

Biochemistry is also concerned with molecular physiology, *i.e.,* the functions of molecules in carrying out the needs of the cells and organs. Again, there is a considerable information in this area and the knowledge about the chemical reactions taking place in cells is increasing rapidly.

Biochemistry in its broad aspects is the most comprehensive of all branches of chemistry. It includes inorganic, analytical, organic and physical chemistry to the extent to which these are related to the chemistry of livings things. The central theme of modern biochemistry, is to correlate the nature, properties and metabolic transformations of chemical constituents of a living system with its morphological structure on one side and its biological functions on the other.

A field of biochemistry, that has stimulated lively interest, is biochemical genetics.

Perhaps the most exciting area of present research in biochemistry deals with mechanisms for the regulations of the synthesis of cellular compounds.

Broadly speaking, biochemistry may be treated as a discipline in which biological phenomena are analysed in terms of chemistry. Due to this reason, the biochemistry is also named as *biological chemistry or chemical biology.*

Modern biochemistry has two separate branches : *descriptive biochemistry and dynamic biochemistry.*

Descriptive biochemistry deals with the qualitative and quantitative characterisation of the various cell components. This branch is more a concern of the organic chemist.

Dynamic biochemistry deals with the elucidation of the nature and the mechanism of the reactions involving the cell components. This branch of biochemistry has now become the language of modern biochemistry.

As the knowledge of biochemistry is increasing rapidly, newer disciplines such as *enzymology* (science of the study of enzymes), *endocrinology* (science of the study of endocrine secretions or the hormones), *clinical biochemistry, molecular biochemistry,* etc., are emerging from the parent biochemistry. Further, *agricultural biochemistry, pharmacological biochemistry,* etc., have also come up as certain link specialities.

Biochemistry shows that living organisms are composed of similar organic compounds, in particular the *proteins and nucleic acids.* These could have been synthesised in conditions prevailing over 4000 million years ago on earth and formed by a process of chemical evolution into a DNA nucleic acid substance capable of replicating itself.

An understanding of the morphological structure of biological material makes biochemistry related to the classical fields of anatomy, histology, pathology, botany, zoology, microbiology and the more modern field of cytology. The need in biochemistry for understanding biological functions makes it also closely related to fields such as physiology, genetics, immunology and endocrinology. To explore the individual characteristics or properties of living system many techniques drafted from and based on the concepts of physics are used. Biochemistry draws so many different areas that it becomes difficult to decide where one ends and where another begins.

Biochemistry is at the base of medicine (including pharmacology to establish structure activity relationship) in the diagnosis and treatment of diseases and possesses important applications in surgery. It has given rise to the science of biochemistry technology, which is making greater contribution to industrial development around the world.

Biochemistry attempts to understand chemical composition, metabolism, morphological structure, biological functions and changes that occur in the living material.

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Advances in biochemistry answers to a series of a major questions like of what chemical compounds are living things composed, what are the structures of macromolecules, characteristic of living system, how do enzymes accomplish their catalytic tasks, what substances are required to satisfy the nutritional requirement of man and other organisms and now the potential energy is available from the oxidation of food stuffs utilised to drive the manifold energy requiring processes of the living cells.

1.2 History of Biochemistry

The term Biochemistry (*Bios* = life) was first of all introduced by a German Chemist Carl Neuberg in 1903.

In terms of history, biochemistry is considered to be a young science. It started largely as an offshoot of organic chemistry and later incorporated ideas obtained from physical chemistry.

Theophrastus Bombastus von Hohenheim (1491–1641), better known as Paracelsus, was the earliest to refer to some chemical aspects of medicine in his writings. First of all, he attained the knowledge of chemistry of his time and then entered the field of medicine to apply his knowledge of chemistry. Later, his followers especially *Jan Baptist van Helmont* (1577–1644) amalgamated the science of chemistry with medicine which appeared under the name of 'medical chemistry' (or latrochemistry).

The basis of biochemistry was, in fact, laid down by chemists like Scheele and Lavoisier. Early chemists were mostly busy in studying the chemistry of plant and animal materials. The Swedish pharmacist *Scheele* (1742–1786) isolated and characterised many substances such as citric acid, lactic acid and uric acid from biological materials.

The classic research done by *Lavoisier* (1743–1794) on oxidation and the role played by the oxygen in the process led him to investigate "burning" in the body and he came to the conclusion that oxygen is consumed in the reaction, carbon dioxide is eliminated, and that heat is evolved. This may be said to be the beginning of modern 'animal calorimetry'. Lavoisier is often spoken of as *'father of modern biochemistry.'*

Wohler (1800-1802) synthesised urea which is the end product of nitrogenous materials in the body. This was an achievement that did much to destroy the notion that animal products were endowed with a vitalism which made them fundamentally different from lifeless substances. The work of Wohler was followed by the synthesis of acetic acid by Adolf Kolbe in 1884 and the synthesis of several organic compounds by Marcellin Berthellot in 1805s. Vitalism was quietly laid to rest. Organic synthesis remains very much alive.

Jestus Von Liebig (1803-1873) has been named as the *'father of agricultural chemistry'*. He showed that "the nutritive materials of all green plants are inorganic substances." He authored many books which offered an impetus in the early development of chemistry. His most interesting book is *'Organic Chemistry in its Applications to Physiology and Pathology,'* which was published in 1842.

At the turn of the century, *Emil Fischer* (1852–1919) ascertained the structure of many carbohydrates, learned to separate amino acids from hydrolysates of proteins, and initiated much of contemporary biochemical though by recognising the optical configurations of carbohydrates and amino acids and by demonstrating the specificity of enzymic action. In postulating the "lock-and-key" concept of enzymic action, Fischer initiated the study of the relation of the topography of macromolecules to the phenomena of life.

Although nucleic acids have been the newest of the four great groups of biochemical materials, their discovery dates back to observations by *Friedrich Miescher* in 1871. His discovery of nueleic acids in the nucleic of pus cells, obtained from discarded surgical bandages, made him to investigate the distribution and the properties of these compounds.

During the first half of the nineteenth century, the studies on heat conducted mainly by *Meyer* and *von Helmholtz* gave rise to the formulation of the 'laws of thermodynamics' which have been essential to the understanding of energy relations in biological systems.

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Besides respiration, the other physiological process which has attracted the attention of biochemists had been that of digestion. Main contributions towards this had been made by *van Helmont, Lazaro Spallanzani* (1729-1799), *Rene de Reaumur, William Beaumont* and *Claude Bernard.* Claude Bernard (1813-1878) of Paris was considered to be the greatest of these. His contributions included the discovery of liver glycogen and its relation to blood sugar in health and disease. He observed the digestive properties of pancreatic juice and did research in muscle and nerve physiology.

The process of fermentation has been probably the single most important process around which the interest of biochemists lasted for a considerable period. By about 1780, fermentation was recognized by *Theodor Schwann* (1810–1882) as a biological process. He ascertained that yeast had been a plant capable of converting sugar to ethanol and carbon dioxide. However, many of the leading chemists of the day, including Berzelius, Wother and Leibing, regarded yeast to be non-living and fermentation to be caused mainly by oxygen. Ridicule by Liebing and Wohler delayed acceptance of Schewann's views until the illustrious French microbiologist *Louis Pasteur* (1822–1895) gave evidence overwhelming all objections. He founded the useful branch of Microbiology in 1857 and was successful in identifying several organisms that carried out various fermentations, including that leading to butyric acid, a type performed by organisms that function without oxygen. He gave definition of fermentation as *"la vie sons l' air"* (life without air). Pasteur, thus, gave the concept of aerobic and anaerobic organisms and their associated fermentations. These conclusions again aroused the ire of Liebing who again took sharp exception but this time with less effect. Such studies on fermentations had been climaxed by the demonstration in 1887 of *Eduard Buchner* (1860–1917) that sugars could be fermented by cell-free extracts of yeast. This discovery is considered to be the cornerstone of much of the enzymological and metabolic study of the twentieth century because it gave rise to technique of isolation and identification that ultimately allowed the study of enzymes and the individual reactions concerned. It also eliminated, yet once and for all, any traces of vitalism for vitalistic theory still lingering.

The extensive researches carried out by *Pasteur* (1822–1895) led *Buchner* (1860–1917) to give modern concept of enzymes. It is to *Michaelis* we owe the present theory of enzyme-substrate inter-actions.

The concepts developed by *Arrhenius, van't Hoff* and *Ostwald* about osmotic pressure and electrolytes and the concept of pH developed by *Sorensen* were proved to be quite useful in understanding the functioning and homeostasis of the body fluids. Later many instruments like Van slyke blood gas apparatus, the ultracentrifuge of Svedberg and the electrophoresis apparatus of Tiselius were discovered. The use of isotopes in biochemical research by *Urey* and Schoenehimer *Synge*, started a new era in modern biochemistry.

Modern biochemistry may be said to have started with the demonstration by *Meyerhof* and *Hill* that there exists a correlation between lactic acid production in contracting muscle and oxygen consumption and heat evolution. The excellent research work carried out by *Warburg, Wieland, Keilin* and *Theorell* led to the discovery of enzymes and cofactors which are involved in cellular oxidation. *Lipmann's* research led to the concept that the radiant energy of the sun utilised by the green plants for the synthesis of foodstuffs is converted in the animal cell into terminal pyrophosphate groups of ATP during the intermediate steps of oxidation.

The significance of unknown food factors was understood by *Frederick Gowland Hopkins* at Cambridge University and his associates, who gave the *concept of deficiency diseases.* Extensive series of feeding experiments utilizing synthetic diets had been carried out mainly by Babcock McCollum, Osborne, Mendel and Sherman. Because of this, many deficiency diseases like scurvy,

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rickets, beriberi and pellagra were recognized and their curative agents, which were called vitamins by a Polish biochemist *Casimer Funk,* has been isolated and subsequently characterized.

The research work done by Szent Gyorgyi and Kreb led to the discovery of sequence of reactions in the fate of lactate during aerobic oxidation. This was termed as Krebs or tricarboxylic acid cycle. It was also proved that the enzymes and co-factors were located in the mitochondrial particles of the cell. Later studies revealed that the fatty acids and amino acids, upon oxidation, also yield intermediates that have been identical with those in the Krebs cycle, thus offering a common mechanism for the liberation of energy from all foodstuffs.

Biochemistry has advanced in recent years on several fronts. The primary polypeptide structure of proteins proposed by Emil Fischer was confirmed by the brilliant researches of Sanger who established the complete amino acid sequence of the protein hormone insulin. du Vigneaud proved the structure of octapeptides of the posterior pituitary by direct synthesis. The studies of Pauling and Corey gave the concept of a secondary structure of protein molecules in the form of a helix.

Waston and Crick proposed the double-stranded structure of DNA molecule. The base sequences in messenger that code for each of the amino acids is now available as a result of researches of Nirenberg, Ochoa and Khorana. The base sequence of transfer RNA molecules specific for different amino acids was ascertained by many workers such as *Holley, Medison, Zachan* and others. A final and accurate list of the base sequences in messenger RNA that code for each of the amino acids was made available because of the brilliant researches by *Nirenberg,* who employed synthetic nucleotides as messenger molecules to identify the code base sequences for each of the amino acids.

Gobind Khorana won Nobel Prize in medicine and physiology in 1968. Now at MIT Khorana is perhaps the foremost expert in nucleotide synthesis having all 64 possible trinucleotide codons. He has also synthesized high molecular weight DNA-like polymers with known sequences and from analysis of proteins they proved that the genetic code is read, consecutively three bases at a time.

A field of biochemistry that has stimulated lively interest is biochemical genetics. The work done by Jacob and Wollman, Muller, Beadle and Tatum established that DNA is coded with respect to its abilities (genes) to direct the synthesis of specific protein.

Perhaps the most exciting area of present research deals with mechanisms for the regulation of the synthesis of important cellular compounds. The phenomenon of feedback inhibition of enzyme activity by the end product of a reaction sequence illustrates a self-regulating mechanism. Enzyme induction and repression—the acceleration or inhibition of synthesis of an enzyme are described. These discoveries give rise to a hypothesis proposed by *Jacob* and *Monod,* suggesting that the DNA molecules consist of areas in which genes have been maintained in an inactive state (by repressors) until they need to get activated for the production of messenger RNA molecules.

One of the most fascinating development in biochemistry during the past decade is the molecular biology which has formed the basis of understanding of secretes of life. Hereditary diseases which were at one time considered to be incurable, are now easily controlled or treated satisfactorily. Studies in this field have given new techniques like recombinant DNA technology or genetic engineering. Recently successful synthesis of complete genome may bring a revolutionary change in the field of biochemistry.

In the 1970s, molecular biology attained a new zenith with the introduction of recombinant DNA technology, which has resulted in a revolution in biological research and has been instrumental in the emergence of a new branch of science called biotechnology. Use of this

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technique in science has helped researchers to isolate eucaryotic genes for studying their structure, regulation and expression.

In this era of biotechnology, biochemistry will occupy a position of central importance. As a basic science, it occupies the foreranks of many scientific endeavours that stand to make a reality of the statement that "the twentieth century belongs to the biologists." It was about 125 years ago that *Hoppe-Seyler* started a journal for those studies in physiological chemistry he believed should be recognized as the new discipline of biochemistry. If he happens to alive today, he would be justifiably proud of the science whose niche he helped define.

1.3 Fields of Research

The achievements of biochemistry and the activities of biochemists can be divided into several large categories.

1. *Kinds and structures of organic compounds :* Fundamental to all other activity has been the compilation of a list of the organic compounds to be found in living organisms together with the elucidation of the structures of each. These myriads of compounds range from the simpler amino acids, sugars, and fatty acids, to the pigments responsible for the colour of flowers, the vitamins and coenzymes, and large macromolecular polymers such as proteins and nucleic acids.
2. *Metabolism and biosynthesis :* The activity that has, perhaps, been most spectacularly successful has been the elucidation of the metabolic path ways for the biosynthesis of naturally occurring compounds from their precursors, that is, from foodstuffs in the case of animals or from carbon dioxide and minerals in the case of plants through photosynthesis. Biochemists have succeeded in establishing much of the details of the major metabolic pathways for the synthesis and degradation of naturally occurring organic compounds in animals, plants, and microorganisms such as bacteria.
3. *Structure and biological function of macromolecules :* A third area of interest relates to a correlation between the structure of macromolecules and their biological function. Thus, biochemists seek to learn how specific proteins serve as catalysts for specific chemical reactions; how the complex polysaccharides of cell walls and membranes serve the cells in which they are found; and how the complex lipids of the nervous system participate in the functions carried out by nervous cells.
4. *Cell function :* Yes another aspect of biochemistry is the search for understanding the way in which the specialized cells of an animal or plant conduct their unique functions such as how muscle cells contract, how certain cells make bone, and how red blood cells transport oxygen and carbon dioxide in the body, and how flowers make their pigments.
5. *Relation of genetics :* Since the 1940s, studies of fungi and bacteria, and later of higher organisms, including man, have shown that genetic mutations often result in the loss of ability to carry out some specific biochemical reaction. These observations led to the formulation of the current concept of a gene as the unit of "information" in a chromosome that directs the biosynthesis of a specific protein. When that protein is an enzyme and the gene has been altered, as in a mutation, the cell loses the ability to conduct the reaction for which enzyme is normally responsible.

   The genes have now been identified as specific portions of molecules of deoxyribonucleic acid (DNA), which can replicate themselves and direct the synthesis of specific protein within the cell. Much of the current work in biochemistry now involves the study of the details of nucleic acid and protein synthesis and is closely related to genetics. Indeed, the two sciences together are often referred to as *molecular biology.*

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6. *Human disease :* No biochemical research is more important than the continuing effort to understand human disease. Every year, increasing numbers of disorders are understood in terms of aberrations from normal pathways of metabolism. Biochemists and physicians have combined to unravel the nature of the fundamental disturbance in such diseases as diabetes and sickle cell disease. More than 800 such metabolic disturbances have been traced to hereditary gene defects, and alleviations for some of these conditions have been found.

Studies of non-genetic conditions have also been important. Investigation of the salt and acid-base composition of blood plasma in health and disease have made possible the practice of major surgery without danger of shock or dehydration, as well as the successful therapy of pernicious vomiting, infantile diarrhoea, and many other disorders.

All diseases are manifestations of abnormalities of molecules, chemical reactions, or processes. The major factors responsible for causing diseases in animals and humans are listed in Table 1.1. All of them affect one or more critical chemical reactions or molecules in the body.

Table 1.1.

THE MAJOR CAUSES OF DISEASES. ALL OF THE CAUSES LISTED ACT BY INFLUENCING VARIOUS BIOCHEMICAL MECHANISMS IN THE CELL OR IN THE BODY

1. **Physical agents** : Mechanical trauma, extremes of temperature, sudden changes in atmospheric pressure, radiation, electric shock.
2. **Chemical agents and drugs** : Certain toxic compounds, therapeutic drugs, etc.
3. **Biologic agents** : Viruses, rickettsia, bacterium fungi, higher forms of parasites.
4. **Oxygen lack** : Loss of blood supply, depletion of the oxygen-carrying capacity of the blood, poisoning of the oxidative enzymes.
5. **Genetic** : Congenital, molecular.
6. **Immunologic reactions** : Anaphylaxis, autoimmune disease.
7. **Nutritional imbalances** : Nutritional deficiencies, nutritional excesses.
8. **Endocrine imbalances** : Hormonal deficiencies, hormonal excesses.

1.4 Aims of Importance of Biochemistry

Whenever a student studies biochemistry, he must have an understanding of the following facts :

(i) *The structures and properties of substances constituting the framework of cells and tissues* : The pioneers of biochemistry showed the presence and nature of substances called proteins, nucleic acids, polysaccharides, and complex lipids. The rate of progress of biochemical understanding has been paced by the development of procedures to isolate and purify such materials. New physiochemical methods showed molecular weights to range from 10000 to more than 100,000,000 for individual substances. For years, the difficult task of establishing the complete structures of such molecules has been found to be experimentally unapproachable. By using, improved analytical techniques and a variety of new physical instruments, including degradative procedures that take advantages of the known specificities of hydrolytic enzymes, the ultracentrifuge, electrophoresis, recording spectrophotometers, spectropolarimeters, amino acid analyzers and X-ray crystallographic analysis, it becomes possible to know the general structure

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of these molecules. Detailed three-dimensional models of several smaller proteins and nucleic acids have been given and there has been rapidly growing understanding of the forces that are able to maintain these molecules, which would otherwise be long, thin, fiber-like structures, folded upon themselves into highly specific compact structures. The biological functioning of these molecules has been found to depend mainly upon their three-dimensional structures.

Understanding of the structures of these large molecules has been rapidly expanding, thereby offering the basis for a more penetrating insight into the mode of operation of enzymes, the structural basis for genetic phenomena, and the fine structure of living cells.

(*ii*) *The structures and properties of substances which enter the cell as useful working materials or sources of energy or leaving the cell as waste products.*

(*iii*) *The catalytic tasks of enzymes* : The study of biochemistry helps us to know how the enzymes accomplish their catalytic tasks. Hundreds of pure enzymes, each more or less specific for one chemical reaction, have been isolated and many crystallized; each has proved to be a distinct, unique protein. This fascinating aspect of science, in many respects forms the heart of biochemistry.

(*iv*) *The chemical processes which convert diet into the compounds which are characteristic of the cells of a given species* : Biochemistry has helped us to know the chemical processes which are used to convert the materials of the diet into the compounds which are characteristic of the cells of a given species. Study of the manifold individual events of metabolism was the centre of biochemical interest.

(*v*) *Using the potential energy obtained from the oxidation of food stuffs utilised to drive the manifold energy-requiring process of the living cell* : The study of biochemistry helps us to know the synthesis of hundreds of new molecular species, intracellular accumulation of inorganic ions and organic compounds against concentration gradients, and the performance of mechanical work. The study of biochemistry also helps us to know the impossibility of utilizing thermal energy to accomplish useful work at constant temperature making untenable a simple analogy between food-burning animals and fuelburning heat engines. By understanding the biological solution to this problem, coupling of the oxidation of carbohydrates and fats to the synthesis of one compound, adenosine triphosphate (ATP), with subsequent utilization of the energy of this compound for virtually all endergonic processes, it has been found to be cardinal to the understanding of living cells.

(*vi*) *Chemistry of inheritance* : Biochemistry helps us to know that it has been the presence, and activities of its complements of proteins that ascertain the form, organisation, and functions of a cell, *i.e.*, its life. It is evident that the genetic "instructions" to a cell must offer the information needed to achieve the precise synthesis of the ensemble of protein characteristic of that cell. This information gets encoded in the structures of the very large molecules of deoxyribonucleic acid (DNA). Cell duplication needs perfect reproduction of these molecules with subsequent equal distribution of the information between the cells. Utilization of this information needs its transmission from nucleus to the ribosome protein factories. Changes in the chemical structure of DNA get evident as mutations observed in subsequent generations. The totality of this information is able to make intelligible the laws of genetics, the nature and basis of hereditary diseases, and the biochemical operation of the process of evolution.

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(*vii*) *The molecular basis of life* : The study of biochemistry helps us to study the molecular basis of life. Some of the main achievements of biochemistry are the discovery of the structure of DNA, the determination of the three-dimensional structure of some protein molecules, and the unfolding of the central metabolic pathways. Organisms as diverse as the bacteria and mammals possess many common features at the molecular level. They employ the similar building units to construct macromolecules. The flow of genetic information from DNA through RNA or protein has been reported to be essentially the same in both types of organisms.

(*viii*) *In diagnosing* : The study of biochemistry plays a vital role in clinical diagnosis. For examples, the level of certain enzymes in the serum reveals many diseases.

(*ix*) *Discovery of new drugs* : The study of biochemistry has helped us to provide a basis for the specific design of drugs.

(*x*) *Solving some fundamental problems in biology and medicine* : The rapid development of biochemistry in recent years has helped scientists involving some of the most challenging and fundamental problems in biology and medicine. For example, how is a single cell able to give rise to cells as different as those in muscle, the kidney and, the brain ? How are cells able to find each other in forming a complex organ. What forms the mechanism of memory ? How does light bring about a nerve impulse in the retina of eye ? What have been the cause of Schizophrenia ? What have been the causes of cancer ? Since the time of *Aristotle*, students of biology try to correlate structure and function, and this endeavour continues. The correlation of biological function and molecular structure forms the main theme of biochemistry.

From the above it follows that the main objective of biochemistry is to fill the wide gap between the highly integrated functions of the living cells and the various properties of its individual chemical constituents. Thus, *a biochemist is an investigator who utilises chemical, physical or biological techniques to study the chemical nature and behaviour of living matter* (The American Society of Biological Chemists, 1965).

1.5 Biochemical Composition of Living Organisms

The basis of all life is the material called protoplasm which can not be defined adequately. It differs from life-less material in possessing the capabilities of growth, repair and reproduction. It is the most complicated physicochemical system known and each form of plant and animal life has a characteristic kind, yet the chemical composition, organisation and chemical processes in these different forms of protoplasm are similar in many respects.

Protoplasm is composed of water and inorganic salts or organic compounds. Water is undoubtedly the most important compound in the tissue and when it is greatly increased or reduced, abnormal physiological functioning occur. The proper distribution of water between the blood, lymph and tissue cells is one of the most important processes of the body. The water balance of the body is closely associated with the concentrations and kinds of electrolytes present, and the proper distribution of the latter is fundamental to the composition and functioning of tissues.

Like all matter found on this planet, living organising are made up of elements which in turn are derived from chemical compounds occurring in living organisms. We will now study the fundamental aspect of chemical composition, namely the elements and organisms of which the organism is composed of.

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1. Element composition : The living organisms are made up of elements. Nearly 50 elements occur in measurable amounts in most living organisms. Among these, the six elements such as oxygen, carbon, hydrogen, nitrogen and phosphorus constitute nearly 99% by weight of a living organism. Another five elements such as sulphur, potassium, sodium, chlorine and magnesium constitute the 0.8% by weight of a living organism. The remaining thirty one elements are present in very small amounts and are known as trace elements. In Table 1.2, the distribution of elements in living organisms has been given.

Table 1.2
ELEMENTAL COMPOSITION OF LIVING ORGANISMS

Elements	*% by weight*
Oxygen (O)	65
Carbon (C)	18
Hydrogen (H)	10
Nitrogen (N)	3
Calcium (Ca)	1.5
Phosphorus (P)	1.0
Sulphur (S)	0.25
Potassium K	0.20
Sodium Na	0.15
Chlorine (Cl)	0.15
Magnesium (Mg)	0.05
Iron (Fe), Zinc (Zn)	Trace amounts
Copper (Cu), Manganese (Mn)	

It is important to remember that the distribution of the elements in living organisms is not the same as their natural abundance in the earth's crust.

2. Chemical composition : All living organisms are made up of different chemical substances but all these chemical substances show similarity in their nature. Nearly 60 to 80% of the weight of an active living organism consists of water. However, there are some metabolically less active tissues which may contain smaller amounts.

The dry weight of cells is composed primarily of four classes of large organic compounds such as carbohydrates, lipids, proteins and nucleic acids. In addition, the cells also contain some small organic molecules and inorganic minerals. Although there occurs considerable variation in the chemical composition of different cells, an average composition may be included in Table 1.3.

Table 1.3
CHEMICAL COMPOSITION OF A CELL

Chemical compound	*Approximate per cent dry weight*
Protein	71
Lipid	12
Nucleic acid	7
Carbohydrate	5
Inorganic minerals and other materials	5

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Table 1.4 lists some details of these compounds.

Table 1.4
MAJOR COMPOUNDS OF LIVING BEINGS

Organic compound	*Building block*	*Some major functions*	*Examples*
Carbohydrate :			
Monosaccharide	—	Energy storage; physical structure	Glucose, fructose, galactose
Disaccharide	Monosaccharides	Energy storage; physical structure	Lactose, maltose, sucrose
Polysaccharide	Monosaccharides	Energy storage; physical structure	Starch, cellulose, chitin, inulin, pectin
Protein	Amino acids	Enzymes; toxins; physical structures	Antibodies; viral surface; flagella; pili
Lipid :			
Triglycerides	Fatty acids and glycerol	Energy storage; thermal insulation; shock absorption	Fat, oil
Phospholipids	Fatty acids, glycerol, phosphate, and an R group*	Foundation for cell membranes	Plasma (cell membranes)
Steroids	Four-ringed structure**	Membrane stability	Cholesterol
Nucleic acid	Ribonucleotides; Deoxyribonucleotides	Inheritance; instructions for protein synthesis	DNA, RNA

*R group = a variable portion of a molecule.
**Technically, steroids are neither polymers nor macromolecules.

1.6 Role of Water in Biological Systems

In all cells, water acts as the medium in which all cellular reactions take place. Thus, water is essential to life and is therefore called *'solvent of life'*.

As water is a good solvent, it is able to dissolve many substances. Because of this property, water finds use to transport nutrients and waste products into and out of cells.

As water has the ability to ionise many of the substances it dissolves, this property of water finds use in several metabolic processes.

Water itself takes part in several reactions either as reactant or product.

Water is having numerous unusual physical properties which make it a unique compound. It is having a much higher specific heat, surface tension, dielectric constant, enthalpy of vaporisation and enthalpy of fusion than other solvents. These unusual properties of water are due to the ability of water molecules to form *hydrogen bonds* among themselves.

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The high specific heat and enthalpy of vaporisation are helpful in the dissipation of the large amounts of heat generated during metabolic activity. Because of this, the temperature fluctuation within the living organism as also in environment is kept to a minimum.

Due to the high dielectric constant of water, it is able to dissolve numerous ionic compounds.

Due to the high surface tension of water, the movement of water occurs in capillary systems.

The maximum density of water is at 4°C but not at 0°C, its freezing point. This unusual property of water is especially important for marine organisms. The result is that ice, being lighter, floats on water instead of sinking and forms an insulating layer. If it had been heavier and sank to the bottom, large areas would have been frozen solid, thereby making life impossible in these areas.

1.7 Biochemistry as Molecular Logic of Living Organisms

Biochemistry while operating at a molecular level and brings to light the hidden secrets of life. Therefore, in studying the molecules of various biological compounds, it becomes essential to

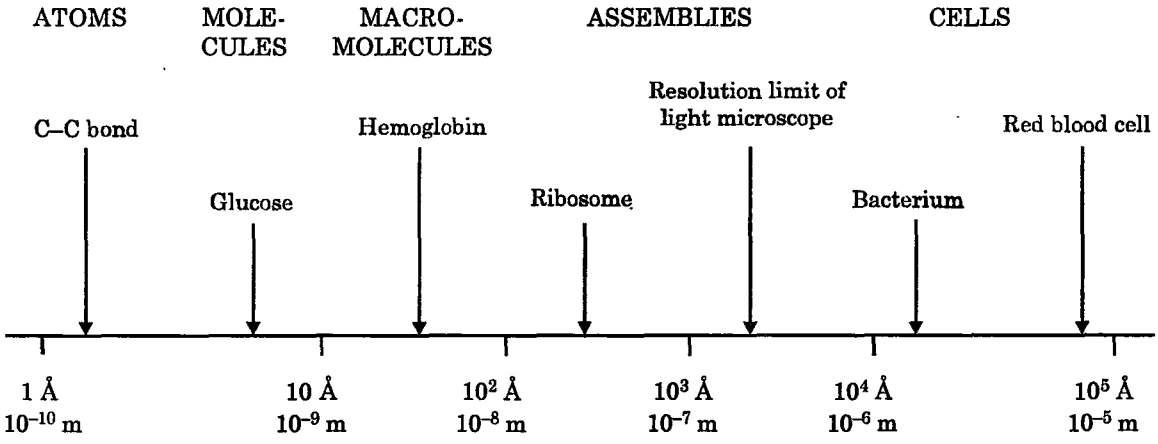

Fig. 1.1. Dimensions of biomolecules, assemblies and cells.

have an idea of scale (Fig. 1.1). The angstrom* (Å) unit (1 Å = 10^{-10} meter or 10^{-8} centimeter or 0.1 nanometer) is generally used as the unit of length at atomic level. Small biomolecules like amino acids, sugars etc., are many angströms long while biological macromolecules are much larger : for example, hemoglobin, an oxygen-carrying protein in red blood cells, is having a diameter of 65 Å. Ribosomes, the protein-synthesizing organelles of the cell, are having diameters of about 300 Å. Most viruses have a range of 100 Å (= 10 nm) to 1,000 Å (= 100 nm). Cells are mostly a hundred times as large. They come in the range of micrometers (μm). For example, a red blood cell has been 7 μm (= 7×10^4 Å) long. The limit of resolution of the light microscope is about 0.2 μm (= 2,000 Å). Therefore, most of the studies of biological structures in the range between 1 Å (= 0.1 nm) and 10^4 Å (= 1 μm) are carried out with electron microscope and x-ray diffraction.

The approximate sizes of the components of the hierarchy of organization in cells are given as follows :

Atoms	Å
Micromolecules (Amino acids)	1 nm
Macromolecules (Proteins)	5 to 550 nm
Organelles	nm to μm
Cells	μm to cm

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The sizes of biomolecules are given in Fig. 1.2. The enzymes convert their substrate into product in milliseconds (1 m sec = 10^{-3} second). Some enzymes are even able to catalyze their substrate in even few microseconds (1 μ sec = 10^{-6} sec). The unwinding of the DNA double helix, is essential for its replication and this gets completed in a microsecond. The rotation of one sphere of a protein with respect to another occurs in nanoseconds (1 n sec = 10^{-9} sec). The primary event in vision — a change in structure of the light-absorbing group – takes place within a few picoseconds (1 p sec = 10^{-12} sec) after absorbing a photon.

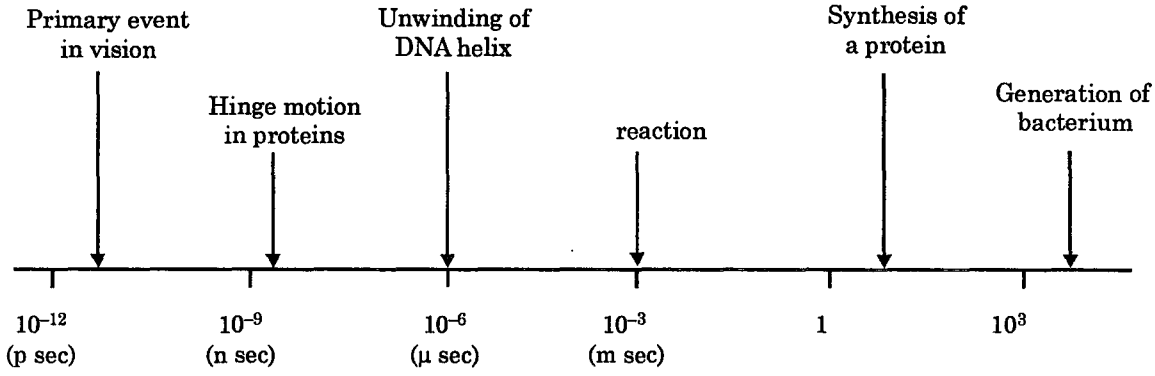

Fig. 1.2. Rates of some biological processes.

The molecular biological events are accompanied by energy changes (Fig. 1.3). The ultimate source of energy for the living objects is the sun. The energy of visible light, say green, has been 57 kilocalories per mole (kcal/mol). ATP, is the universal currency of energy, and is having a usable energy content of about 12 kcal/mol. The amount of energy required for the cleavage of a covalent C—C bond has been 83 kcal/mol. Hence, the covalent skeleton of biomolecules is stable in the absence of enzymes and inputs of energy. On the other hand, thermal energy is sufficient to make and break noncovalent bonds in living systems, which typically are having energy contents of only a few kilocalories per mole.

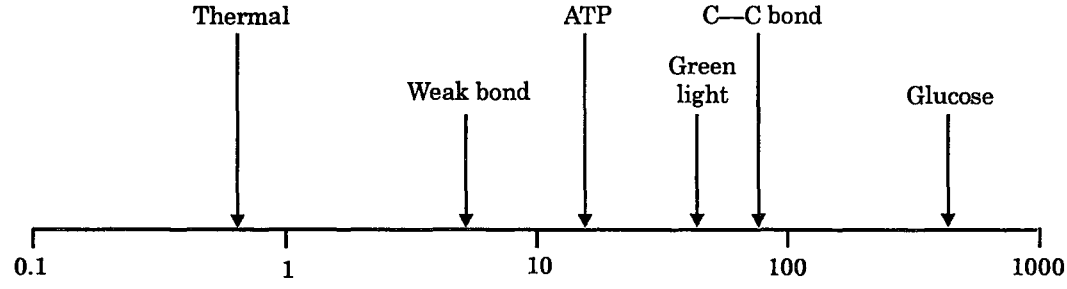

Fig. 1.3. Some biologically important energies have been shown.

1.8 Metabolism

Though, in nature, living organisms are characterized by a constancy of size, shape, weight and composition, they are not in a static condition and there is a constant breakdown turnover and resynthesis of the macromolecular components or organisms. Man and other organisms ingest as foodstuff a complex mixture of plant and animal carbohydrates, fats and proteins. During digestion, these are broken down into a small number of simples substances—glucose, amino

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acids, fatty acids and glycerol. The smaller molecules of *metabolites* enter the various cells of the body *via* the blood stream and become the raw materials of maintenance, growth and development.

The sum total of all the enzymatic reactions occurring in cell is collectively called *metabolism* (Greek-metabolos means "Changeable"). The reaction sequences occurring within organisms in an orderly and regulated way are known as *metabolic pathways*. Because metabolism proceeds in a stepwise manner through many intermediates, the turn intermediary metabolism is often used to denote the chemical pathways of metabolism.

Metabolism has different aspects. Reactions comprising the total metabolism of any cell can be broadly divided into two types—degradative and synthetic. The degradative reactions are collectively referred to as *catabolism* and the synthetic reactions as *anabolism*. Catabolic reactions are oxidative-involve the oxidation of the carbon atoms of carbohydrates, lipids and proteins. Anabolic reactions, on the other hand, are reductive and involve the biosynthesis of various molecules in the organism. As a result of oxidation of carbon atoms useful chemical energy is released (ΔG is –ve); by contrast, biosynthetic processes are energy requiring (ΔG is + ve). In catabolism the chemical energy of metabolites is conserved in the form of ATP whereas in biosynthetic pathways ATP is utilized. In living systems catabolism and anabolism occur concurrently and simultaneously in cells. The energy released during catabolic processes is required, apart for biosynthesis, for many cellular activities like muscular contraction, nervous conduc-tion, absorption and secretion.

Stages of Metabolism

Catabolic and anabolic changes take place in three stages. In stage I of catabolism large nutrient molecules are degraded into their major building blocks. In stage II, the different products of stage I are converted into a smaller number of still simpler intermediates. Thus, carbohydrates are converted into pyruvate and then to acetyl-SCoA. Similarly, fatty acids and amino acids are converted to acetyl-SCoA and some other products. Stage III is a common catabolic pathway in which products of stage II are ultimately oxidized to carbon dioxide and water. During

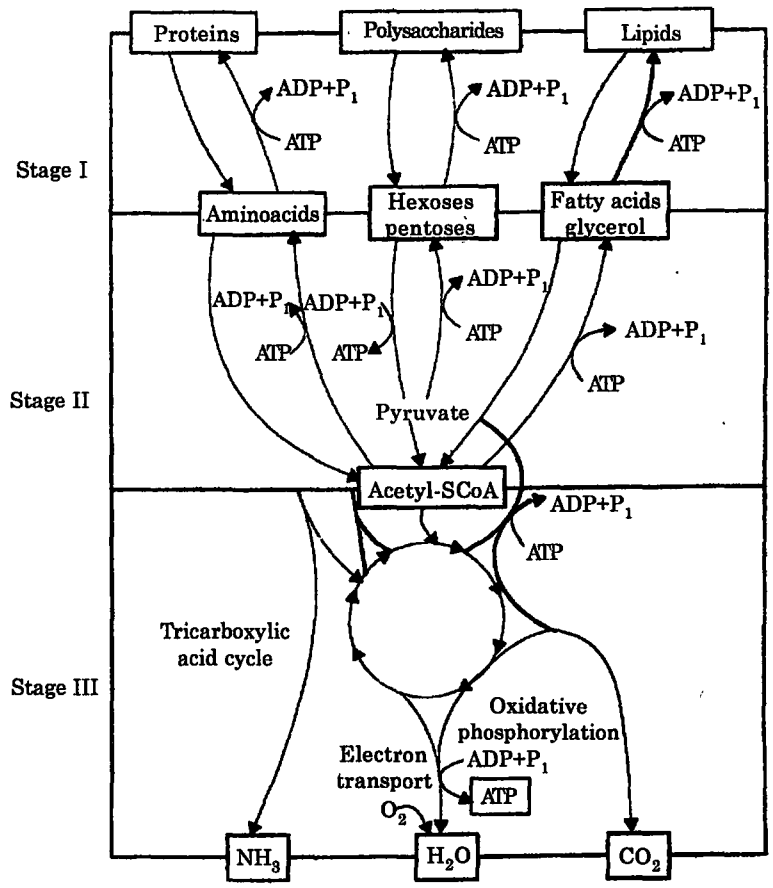

Fig. 1.4. The three stages of catabolism and anabolism.

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anabolism, the intermediates formed in stage III are converted into building block molecules of stage II which are finally assembled into macromolecules of stage I. The stages of metabolism are shown in Fig. 1.4.

1.9 Metabolic Mill (Fig. 1.5)

Chemical changes in living organisms usually occur in a series of steps which are referred to as *metabolic pathways*. The compounds formed in this series of reactions are called *intermediates*. Metabolic pathways have been classified as *anabolic, catabolic and amphibolic.* In *anabolic* or biosynthetic pathways, a relatively simple compound is built up into a more complex molecule. Such pathways therefore build up or synthesize cell constituents. *Catabolic* or degradative pathways, on the other hand, result in the breakdown of compounds or decrease in molecular complexity. Relatively high molecular weight compounds are degraded to lower molecular weight compounds. Energy released during catabolism is often stored in the form of ATP or some other high energy compounds. The products of a catabolic pathway are excreted if toxic. Some products may be diverted into anabolic pathways. In some cases it is difficult to decide whether a pathway is anabolic or catabolic. Such pathways have been termed as *amphibolic* (*or diabolic*).

The energy-yielding pathways of glycolysis, pyruvate oxidation and the Krebs cycle are sometimes referred to as catabolic or degradative pathways. However, several biosynthetic pathways branch off from the intermediates of these pathways. The pathways are therefore better designed as amphibolic. The term catabolic would apply to the short sequences that convert amphibolic intermediates to fermentation end products. The pathways from the amphibolic intermediates to the end products would be the purely anabolic pathways.

The breakdown products of the three main classes of foods, carbohydrates, proteins and fats, can participate in glycolysis and the Krebs cycle. These products exist in

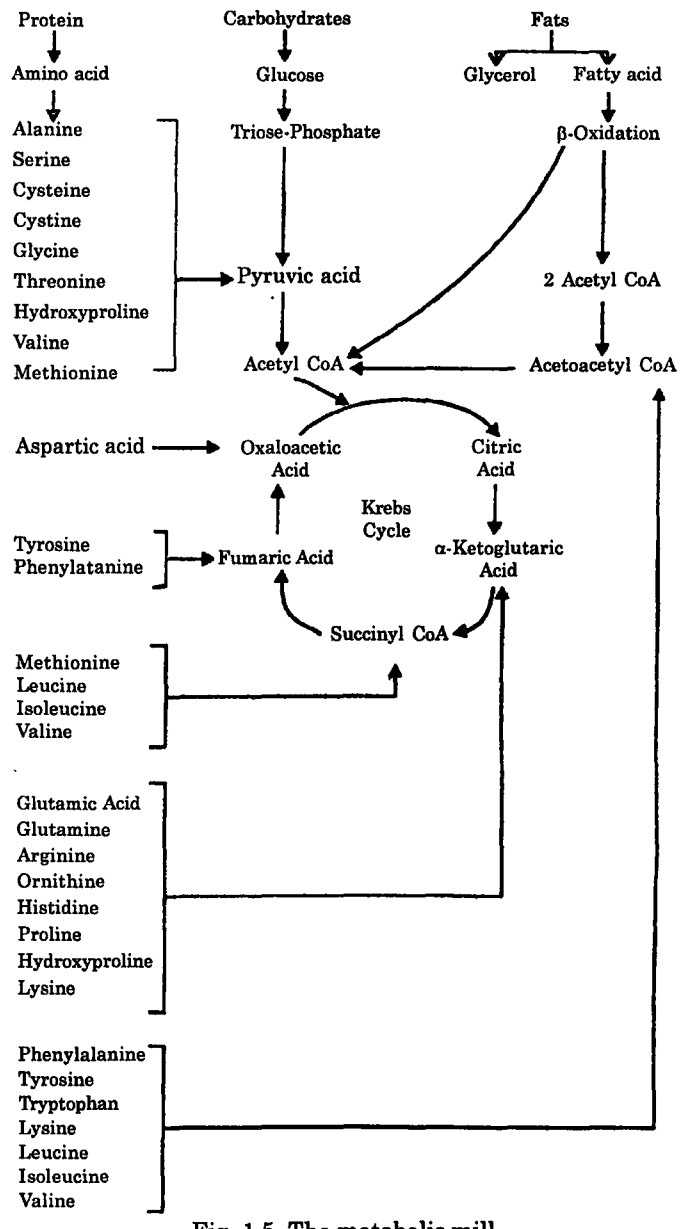

Fig. 1.5. The metabolic mill.

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'metabolic pools' in the cytoplasm, and can be utilized for cell respiration. Thus, interconversion of protein, carbohydrates and fats can occur through the glycolytic pathway and the Krebs cycle (Fig. 1.5). This metabolic interconversion is called the *metabolic mill.*

(1) *Carbohydrates* (polysaccharides) are hydrolysed during the process of digestion to form *monosaccharides.* Of these, the hexose sugar *glucose* is the key compound in respiration. Other sugars like *galactose* and *fructose* can also serve as sources of energy, and are readily convertible into glucose. Glucose is metabolized through the glycolytic pathway and Krebs cycle to yield energy in the form of ATP.

(2) *Proteins* are digested into their constituent amino acids. These amino acids undergo *deamination* and enter at various points, in the glycolytic pathway and the Krebs cycle. During deamination, the amino ($-NH_2$) group of the amino acid is removed. Thus, *alanine* on deamination forms *pyruvic* acid, and *glutamic acid* forms α-*ketoglutaric acid* (Fig. 1.6).

The amino acids alanine, serine, cysteine, cystine, glycine, threonine, hydroxyproline, valine and methionine enter the glycolytic pathway at the *pyruvic acid* stage.

Aspartic acid is converted into *oxaloacetic acid,* and enters the Krebs cycle.

Methionine, leucine, isoleucine and valine enter the Krebs cycle at the *succinyl Co A stage.*

Glutamic acid, glutamine, arginine, ornithine, histidine, proline, hydroxyproline and lysine enter the Krebs cycle at the α-*ketoglutaric acid stage.*

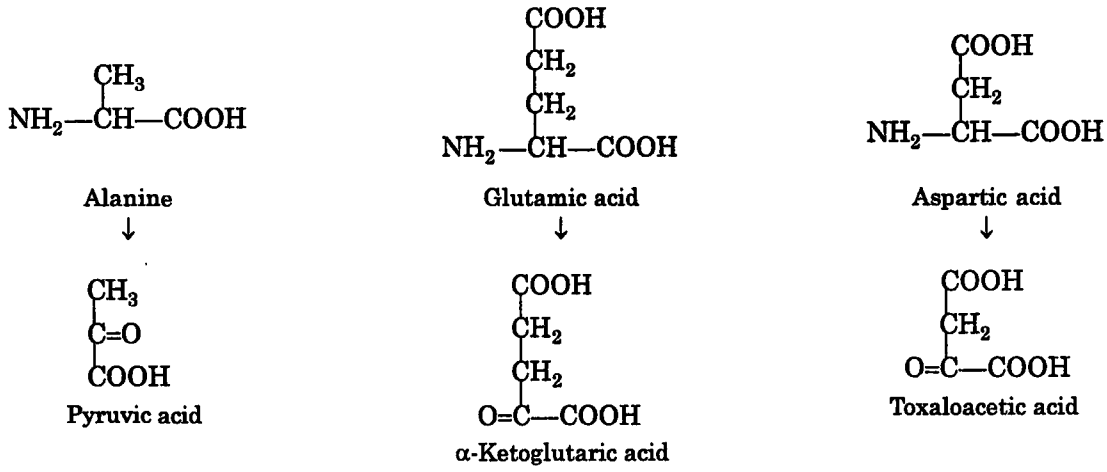

Fig. 1.6. Deamination of amino acids.

Phenylalanine, tyrosine, tryptophan, lysine, leucine and isoleucine enter as *acetoacetyl CoA.*

The pathway can be reversed under certain-conditions. Thus by addition of an amino ($—NH_2$) group (*amination*) α-ketoglutaric acid forms *glutamic acid.* Such amino acids which are synthesized by the cell are called *non-essential amino acids.* They are formed by the cell itself, and need not be taken in the food. On the other hand amino acids which are not synthesized by the cell, must be obtained from food, are called *essential amino acids.*

(3) *Fats* on digestion are broken down to *glycerol and fatty acids.* Glycerol enters the glycolytic pathway at the *triose phosphate stage.*

Fatty acids undergo *B-oxidation,* by which two carbon fragments of acetyl Co A are split off at a time from the fatty acid chain. This ultimately results in complete oxidation of the fatty acid.

It will thus be seen that the three basic organic substances of the cell can be manufactured by interconversion through the glycolytic pathway and the Krebs cycle. The cell is therefore not

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entirely dependent on outside sources for its materials. Under conditions of carbohydrate scarcity, the breakdown products of proteins and fats can also be utilized for providing energy to the cell. When carbohydrates are in excess, fats can be synthesized from them. Thus eating of excess of potatoes (carbohydrates) results in fatness. Conversely, after prolonged fasting a person becomes thin, as his fat reserves are utilized for cell respiration. This permits a degree of flexibility in the biochemical system of the cell.

1.10 Health

The World Health Organisation (WHO) defines health as a state of "complete physical, mental and social well-being and not merely the absence of disease and infirmity." From a strictly biochemical view-point, *health* may be considered *that situation in which all of the many thousands of intra-and extra-cellular reactions that occur in the body are proceeding at rates commensurate with its maximal survival in the physiologic state.*

Normal biochemical processes are the basis of health.

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2 Concepts of Cell

2.1 Introduction

In 1665 Robert Hooke, using a primitive microscope first described how cork was composed of 'chambers' or 'cells.' Ten years later, Anton van Leeuwenhoek made his historical observations of micro-organisms, including what we now call *Protozoa* and bacteria. The use of stains to enhance the contrast between microscopic structures and their background, the improved sectioning of material, and advances in optics, made possible the description of many plant and animal tissues in the early nineteenth century. This work culminated in the *cell theory* (Schleiden and Schwann, 1838-39).

2.2 Cell Theory

(*i*) *All living organisms are composed of one or more units called cells.*

A working definition of a cell is that it is a unit of *protoplasm* surrounded by a thin *plasma membrane.*

The protoplasm of both animal and plant cells usually contains an approximately spherical object, the *nucleus*. Such cells are described as *eukaryotic* (true nucleus) to distinguish them from the *prokaryotic* (non-nucleate) bacteria and blue-green algae. Some of the other important differences which exist between eukaryotes and prokaryotes are indicated in Table 2.1.

The rest of the protoplasm is called the *cytoplasm*. Using appropriate staining and microscopic techniques, the cytoplasm is seen to contain many subcellular structures called *organelles*. Some

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of these are *membrane bound*, others are *fibrillar*. The *cytosol* (syn. ground matrix, hyaloplasm) in which the structures are embedded is rather gelatinous, mainly on account of the proteins present, a proportion of which exist as *colloids*. Amongst the proteins of the cytosol are several important enzyme systems, for example those of *glycolysis*. Many organic substances and mineral ions are also present. Some of these greatly affect the solubility of proteins. Raising the Ca^{2+} level, for example, can convert cytosol from a semi-contracted *gel* to a more liquid, relaxed *sol*. The edge of the cell (*ectoplasm*) is often in the former state, and may appear almost transparent due to the exclusion of larger organelles by the fibrillar system. The interior of the cells is usually a granular sol. (*endoplasm*).

Whilst organelles may vary from one cell to another, one structure which defines the cell absolutely is the *plasma membrane*. The existence of a living cell without one is inconceivable.

No generalisation is universally true, and there are important exceptions to the description of a cell given above. For example, in plant cells the plasma membrane is surrounded by a *cell wall*. By common agreement a plant cell is therefore defined as protoplasm plus cell wall. Again, some cells lack a nucleus altogether, whereas others have more than one. Finally, in some mature plant cells such as xylem vessels, the protoplasm disappears completely, and the term 'cell' is applied to the cell wall only.

The term 'living jelly' as a synonym for protoplasm is best avoided. It suggests that there is some 'vital' force in a cell giving it life. This view (vitalism) lacks the support of experimental evidence and is only of historical interest. Experimental evidence suggests that the activities in a cell which we call 'life' are best explained in terms of the collective physical and chemical properties of the various parts.

(*ii*) *Cells are the basic functional units of tissues, organs and organisms.*

Cells are the essential working components of every structure in an organism. Parts of organisms which are not composed of cells are difficult to find, although parts of bone and the exoskeleton of insects are two examples. In both cases, however, the material is secreted by cells, so it is of cellular origin.

Whilst cells are the fundamental building blocks of tissues and organs, they do not work in isolation. Recent technical developments have considerably advanced our understanding of how cells communicate with each other to produce a unified and coordinated organism.

(*iii*) *Cells only come from pre-existing cells.*

A cell may divide to produce two or, occasionally, more daughter cells. Alternatively a cell such as a zygote may arise from a fusion of two cells. In any event, cells never arise *de novo*.

2.3 Status of the Cell Concept

Scientific theories are inventions of the human mind and nature is under no obligation whatever to obey them. If she chooses not to, we dismiss it as an 'exception.' However, the cell theory is remarkably comprehensive, and about the nearest we get to an exception are the viruses. However, it can be argued that viruses are best regarded as non-living, self-replicating particles, not living organisms. If this view is correct, then there are no exceptions to the cell theory.

2.4 Prokaryotes

Introduction : Prokaryotes (bacteria and blue-green algae) are the most abundant organisms on earth. A prokaryotic cell does not contain a membrane-bound nucleus. Bacteria are either cocci, bacilli or spirilla in shape, and fall into two groups, the eubacteria and the archaebacteria. The eubacteria are the commonly encountered bacteria in soil, water and living in or on larger

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organisms, and include the Gram-positive and Gram-negative bacteria, and cyanobacteria (photosynthetic blue-green algae). The archaebacteria grow in unusual environments such as salt brines, hot acid springs and in the ocean depths, and include the sulfur bacteria and the methanogens.

1. Cell structure : Like all cells, a prokaryotic cell is bounded by a *plasma membrane* that completely encloses the cytosol and separates the cell from the external environment. The plasma membrane, which is about 8 nm thick, consists of a lipid bilayer containing proteins. Although prokaryotes lack the membranous subcellular organelles characteristic of eukaryotes, their plasma membrane may be infolded to form *mesosomes* (Fig. 2.1). The mesosomes may be the sites of deoxyribonucleic acid (DNA) replication and other specialized enzymatic reactions. In photosynthetic bacteria, the mesosomes contain the proteins and pigments that trap light and generate adenosine triphosphate (ATP). The aqueous cytosol contains the macromolecules [enzymes, messenger ribonucleic acid (mRNA), transfer (*t*RNA) and ribosomes], organic compounds and ions needed for cellular metabolism. Also within the cytosol is the prokaryotic 'chromosome' consisting of a single circular molecule of DNA which is condensed to form a body known as the *nucleoid* (Fig. 2.1). Many bacterial cells have one or more tail-like appendages known as *flagella* which are used to move the cell through its environment.

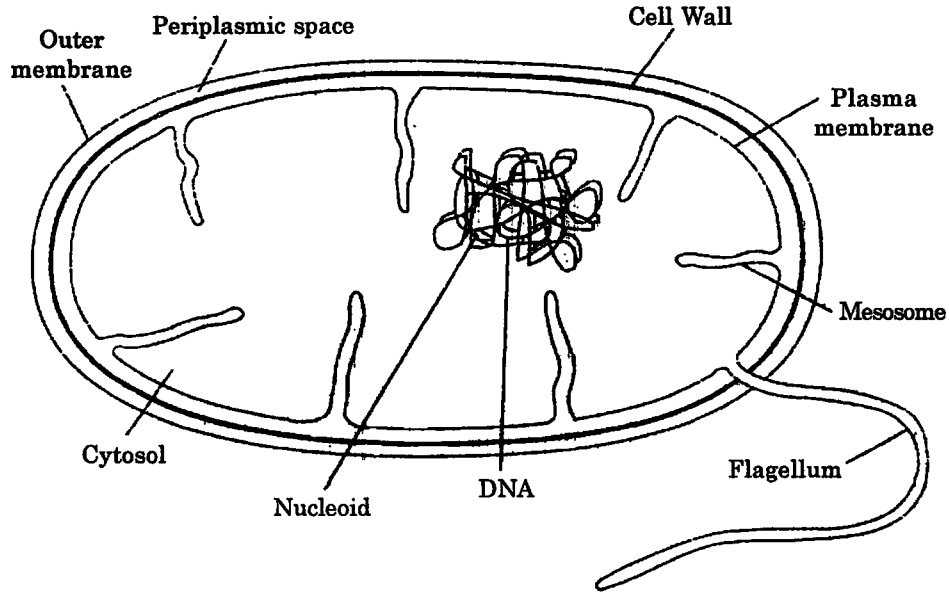

Fig. 2.1. Prokaryote cell structure.

2. Bacterial cell wall : To protect the cell from mechanical injury and osmotic pressure, most prokaryotes are surrounded by a rigid 3–25 nm thick *cell wall* (Fig. 2.1). The cell wall is composed of *peptidoglycan*, a complex of *oligosaccharides* and *proteins*. The oligosaccharide component consists of linear chains of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (NAM) linked β(1–4). Attached via an amide bond to the lactic acid group on NAM is a *D-amino acid*—containing tetrapeptide. Adjacent parallel peptidoglycan chains are covalently cross-linked through the tetrapeptide side-chains by other short peptides. The extensive cross-linking in the peptidoglycan cell wall gives it its strength and rigidity. The presence of D-amino acids in the peptidoglycan renders the cell wall resistant to the action of *proteases* which act on the more commonly occurring L-amino acids, but provides a unique target for the action of certain *antibiotics*

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such as *penicillin*. Penicillin acts by inhibiting the enzyme that forms the covalent cross-links in the peptidoglycan, thereby weakening the cell wall. The β(1–4) glycosidic linkage between NAM and GlcNAc is susceptible to hydrolysis by the enzyme *lysozyme* which is present in tears, mucus and other body secretions.

Bacteria can be classified as either *Gram-positive* or *Gram-negative* depending on whether or not they take up the *Gram stain*. Gram-positive bacteria (*e.g., Bacillus polymyxa*) have a thick (25 nm) cell wall surrounding their plasma membrane, whereas Gram-negative bacteria (*e.g., Escherichia coli*) have a thinner (3 nm) cell wall and a second *outer membrane* (Fig. 2.2). In contrast with the plasma membrane, this outer membrane is very permeable to the passage of relatively large molecules (molecular weight > 1000 Da) due to *porin proteins* which form pores in the lipid bilayer. Between the outer membrane and the cell wall is the *periplasm*, a space occupied by proteins secreted from the cell.

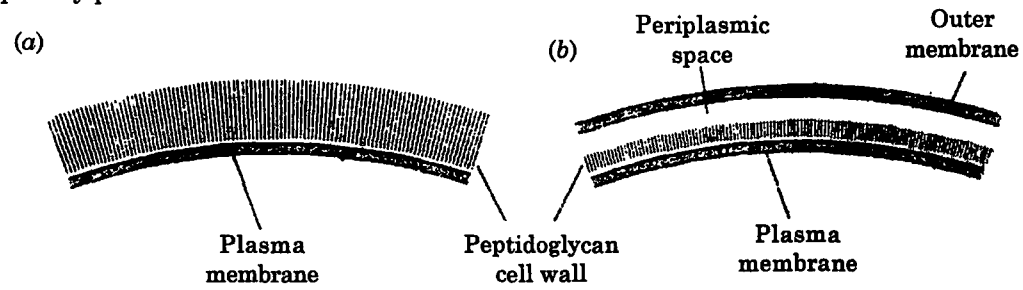

Fig. 2.2. Cell wall structure of (*a*) Gram-positive and (*b*) Gram-negative bacteria.

2.5 Eukaryotes

A eukaryotic cell is surrounded by a *plasma membrane*, has a membrane-bound nucleus and contains a number of other distinct *subcellular organelles* (Fig. 2.3). These organelles are membrane-bounded structures, each having a unique role and each containing a specific complement of proteins and other molecules. Animal and plant cells have the same basic structure, although some organelles and structures are found in one and not the other (*e.g.*, chloroplasts, vacuoles and cell wall in plant cells, lysosomes in animal cells).

1. Plasma membrane : The plasma membrane envelops the cell, separating it from the external environment and maintaining the correct ionic composition and osmotic pressure of the cytosol. The plasma membrane, like all membranes, is impermeable to most substances but the presence of specific proteins in the membrane allows certain molecules to pass through, therefore making it *selectively permeable*. The plasma membrane is also involved in communicating with other cells, in particular through the binding of ligands (small molecules such as hormones, neurotransmitters, etc.) to *receptor proteins* on its surface. The plasma membrane is also involved in the *exocytosis* (secretion) and *endocytosis* (internalization) of macromolecules.

2. Nucleus : The nucleus stores the cell's genetic information as DNA in chromosomes. It is bounded by a double membrane but pores in this membrane allow molecules to move in and out of the nucleus. The nucleolus within the nucleus is the site of ribosomal ribonucleic acid (rRNA) synthesis.

3. Endoplasmic reticulum : This interconnected network of membrane vesicles is divided into two distinct parts. The rough endoplasmic reticulum (RER), which is studded with ribosomes, is the site of membrane and secretory protein biosynthesis and their post-translational modification. The smooth endoplasmic reticulum (SER) is involved in phospholipid biosynthesis and in the detoxification of toxic compounds.

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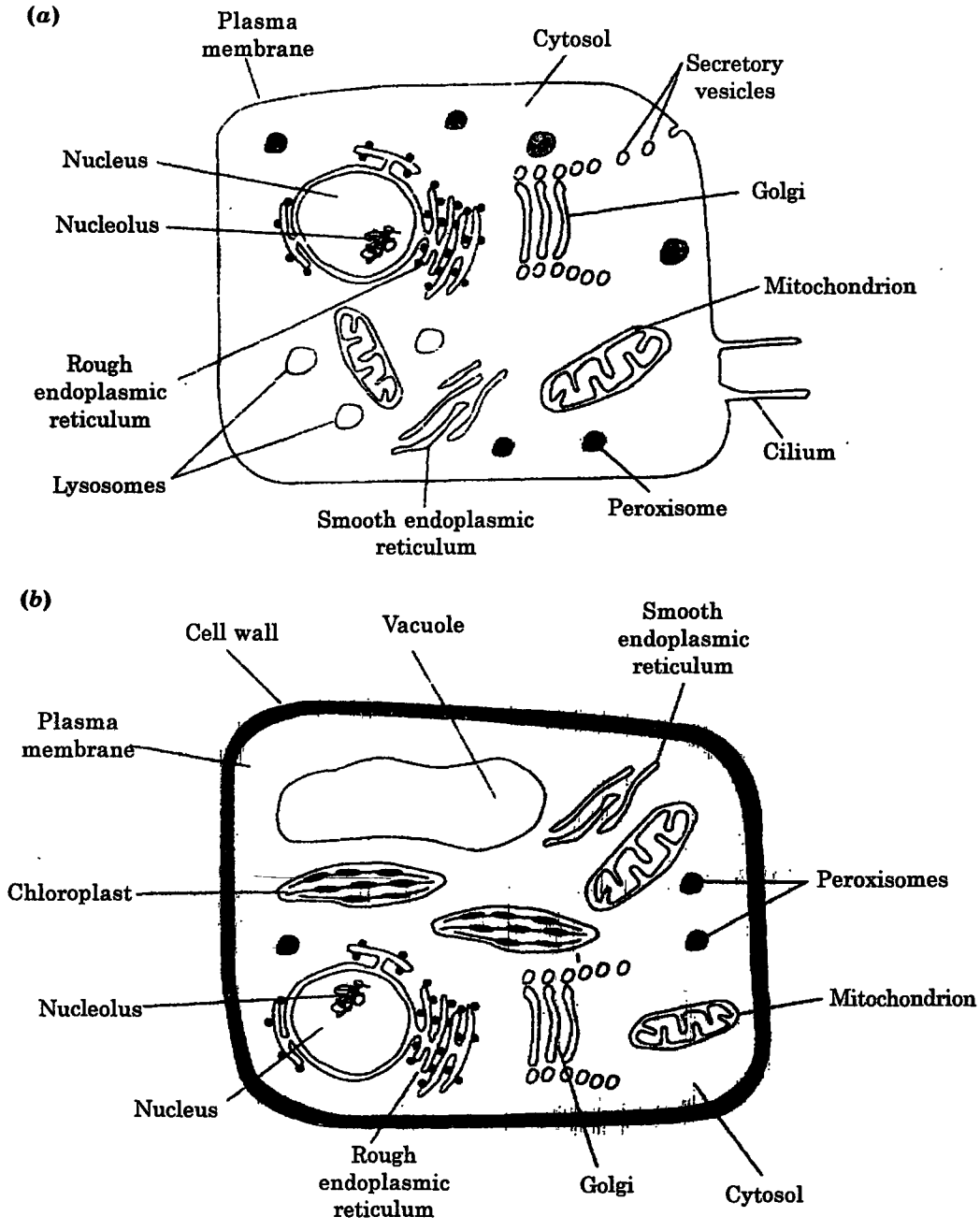

Fig. 2.3. Eukaryote cell structure. (*a*) Structure of a typical animal cell, (*b*) Structure of a typical plant cell.

4. Golgi apparatus : The Golgi apparatus, a system of flattened membrane-bound sacs, is the sorting and packaging center of the cell. It receives membrane vesicles from the RER, further modifies the proteins within them, and then packages the modified proteins in other vesicles which eventually fuse with the plasma membrane or other subcellular organelles.

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5. Mitochondria : Mitochondria have an inner and an outer membrane separated by the intermembrane space. The outer membrane is more permeable than the inner membrane due to the presence of porin proteins. The inner membrane, which is folded to form cristae, is the site of oxidative phosphorylation, which produces ATP. The central matrix is the site of fatty acid degradation and the citric acid cycle [Fig. 2.4 *a*].

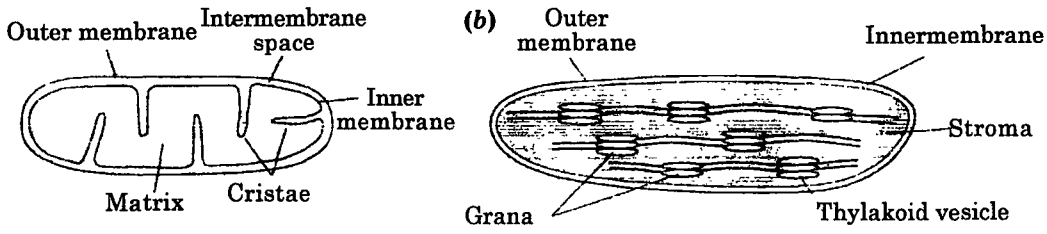

Fig. 2.4. Structure of (*a*) a mitochondrion and (*b*) a chloroplast.

6. Chloroplasts : Chloroplasts also have *inner and outer membrane.* In addition, there is an extensive internal membrane system made up of *thylakoid vesicles* (interconnected vesicles flattened to form discs) stacked upon each other to form *grana* [Fig. 2.4 (*b*)]. Within the thylakoid vesicles is the green pigment *chlorophyll,* along with the enzymes that trap light energy and convert it into chemical energy in the form of ATP. The *stroma,* the space surrounding the thylakoid vesicles, is the site of carbon dioxide (CO_2) fixation—the conversion of CO_2 into organic compounds. Chloroplasts, like mitochondria, contain DNA which encodes some of the chloroplast proteins.

7. Lysosomes : Lysosomes in animal cells are bounded by a single membrane. They have an acidic internal pH (pH 4–5), maintained by proteins in the membrane that pump in H^+ ions. Within the lysosomes are acid hydrolases; enzymes involved in the degradation of macromolecules, including those internalized by endocytosis.

8. Peroxisomes : Peroxisomes contain enzymes involved in the breakdown of amino acids and fatty acids, a byproduct of which is hydrogen peroxide. This toxic compound is rapidly degraded by the enzyme catalase, also found within the peroxisomes.

9. Cytosol : The cytosol is the soluble part of the cytoplasm where a large number of metabolic reactions take place. Within the cytosol is the cytoskeleton, a network of fibers (microtubules, intermediate filaments and microfilaments) that maintain the shape of the cell.

10. Cytoskeleton : Eukaryotic cells have an internal scaffold, the cytoskeleton, that controls the shape and movement of the cell. The cytoskeleton is made up of actin microfilaments, intermediate filaments and microtubules.

11. Microtubules : Microtubule filaments are hollow cylinders made of the protein tubulin. The wall of the microtubule is made up of a helical array of alternating α- and β-tubulin subunits. The mitotic spindle involved in separating the chromosomes during cell division is made of microtubules. Colchicine inhibits microtubule formation, whereas the anticancer agent, taxol, stabilizes microtubules and interferes with mitosis.

12. Plant cell wall : The cell wall surrounding a plant cell is made up of the polysaccharide cellulose. In woody plants, the phenolic polymer called lignin gives the cell wall additional strength and rigidity.

13. Plant cell vacuole : Plant cells usually contain one or more *membrane-bounded vacuoles.* These are used to store nutrients (*e.g.,* sucrose), water, ions and waste products (especially excess nitrogen-containing compounds). Like lysosomes in animal cells, vacuoles have an *acidic* pH maintained by H^+ pumps in the membrane and contain a variety of *degradative enzymes.* Entry of water into the vacuole causes it to expand, creating hydrostatic pressure (*turgor*) inside the cell which is balanced by the mechanical resistance of the cell wall.

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2.6 Differences between Prokaryotic and Eukaryotic Cells

Prokaryotic and eukaryotic cells are the two broad classifications of all cells, with significant differences between the two classes.

Prokaryotic cells are very small, simple cells at a primitive level of development. All bacteria and a type of algae (blue-green) are prokaryotic cells. These cells possess a single membrane surrounding the cell, no clearly defined nucleus, and no internal membranes separating internal structures.

Eukaryotic cells comprise the higher plants and animals. These cells have cell membranes and distinct nuclei as well as internal structure surrounded by membranes. These structures, collectively called *organelles,* include mitochondria, Golgi bodies, endoplasmic reticulum, and chloroplasts (in green plants).

Table 2.1
COMPARISON OF EUKARYOTIC AND PROKARYOTIC CELLS

	Eukaryotes (animal/plant cells) (Eu : true; karyon : nucleus)	*Prokaryotes (bacteria/blue-green algae) (Pro : without; karyoon : nucleus)*
1. Nucleus	Present	Absent
2. Nucleolus	Present	Absent
3. DNA	Confined mostly to several linear nuclear organelles (chromosomes)	Confined mostly to a single circular loop (chromoneme) attached to the plasma membrane
4. DNA packaging	Histones (proteins) help package DNA into chromosomes	Histones absent
5. Cell wall (plants only)	Mostly cellulose, hemicellulose, pectate	Never cellulose, several unique polymers (murins/techoic acid)
6. Plasma membrane	Phospholipids, sterols, proteins	Phospholipids and proteins, no sterols
7. Membrane-bounded systems	Chloroplasts (Plants), mitochondria, E.R., Golgi bodies, lysosomes, etc.	None. Photosynthetic pigments (present) in infoldings plasma membrane
8. Cilia/flagella	9 + 2 system of microtubules	If present, superficially resemble single microtubule, but made of a unique protein
9. Internal cytoskeletal components	Extensive microtubules, microfilaments, 10 nm filaments	None
10. Ribosomes	80S (larger)	70S (smaller)
11. Storage compounds	Often glycogen (animals) or starch (plants)	Varied. Often a polymerised fatty acid derivative, β-hydroxybutyrate (bacteria); sometimes glycogen (blue-green algae). Not starch (except *Clostridium*)
12. Organisation	Cells mostly components of organs and tissues in a complex multicellular organism	Unicells, or short chains of similar cells
13. Size	Varied, typically $10^4\ \mu m^3$ to $10^5\ \mu m^3$	Varied, typically 1 μm^3 to 10 μm^3

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2.7 Cellular Organisation

As we have seen above, eukaryotic cells have compartmentalization of organelles by membranes. This compartmentalization is important in *metabolism*—the breakdown of food materials (*catabolism*) and synthesis of new cell components (*anabolism*). In eukaryotic cells, certain metabolic enzymes are located in specific organelles or cellular sites. For example, the mitochondria contain all the enzymes for the electron transport system (ETS) used for ATP production. Commonly, a sequence of reactions is catalyzed by a sequence of specific enzymes. In these cases, it is likely that the enzymes are located in the organelle so that they are adjacent in the order required by the reaction sequence.

By separation of enzyme systems, opposing chemical reactions can be occurring simultaneously in different cellular locations. For example, the breakdown of fatty acids can occur at one cellular site and at the same time fatty acid synthesis takes place elsewhere. Synthesis-catalyzing enzymes are compartmented and separated from the site where enzymes catalyzing fatty acid breakdown occur.

This does not mean to imply that subcellular components act independently. In a cell there exists a finely tuned coordination among subcellular components and the cell's external environment to meet the metabolic needs of the cell.

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3 Biophysical Processes

3.1 Water

Water is a vital substance. A 70 kg man contains approximately 45 dm^3 (litres) of water and his death will ensure when about 20% of the body water is lost. Varying quantities of water are stored in different body tissues where it is not slopping about as in a bucket but is compartmentalized by cell membranes so that it is a major constituent of the cytosol. Water comes into contact with and bond to cellular components, *e.g.*, proteins, nucleic acids and lipids, and in this way, cellular water can and does affect the properties of biological compounds.

The water molecule is an irregular tetrahedron with oxygen at its center (Fig. 3.1). The 2 bonds with hydrogen are directed toward 2 corners of the tetrahedron, while the unshared electrons on the 2 sp^3 hybridized orbitals occupy the 2 remaining corners. The angle between the 2 hydrogen atoms (105 degrees) is slightly less than tetrahedral angle (109.5 degrees), forming a slightly skewed tetrahedron.

Because of its skewed tetrahedral structure electrical charge is not uniformly distributed about the water molecules. The side of the oxygen opposite to the 2 hydrogens is relatively rich in electrons, while on the other side the relatively unshielded hydrogen nuclei form a region of local positive charge. The term *dipole* denotes molecules such as water that have electrical charge (electrons) unequally distributed about their structure.

The physical properties of water are markedly different from those of other commonly used solvents. Water has a higher melting point, boiling point, heat of vaporization (the amount of

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heat energy required to change 1 g of water from its liquid state into the gaseous state, expressed in Jg^{-1}) and dielectric constant (the capacity to store electrical potential energy in an electric field). These higher values are due to the structure of water. Each O—H bond is formed by a sharing of electrons and is therefore a covalent bond. However, the more electropositive oxygen nucleus attracts the electrons more strongly than the hydrogen atom which results in a slight negative charge (δ^-) in the region of the oxygen atom and a light positive charge (δ^+) in the region of the hydrogen atom. This means that the water molecule, although it has no net charge, has two regions of partial positive and one region of partial negativity which results in the molecule being an electronic dipole, *i.e.*, polar.

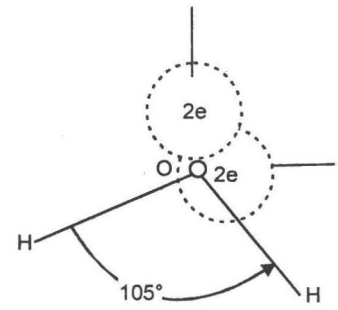
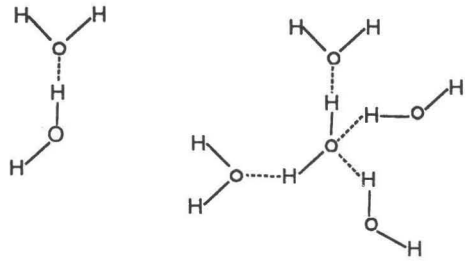


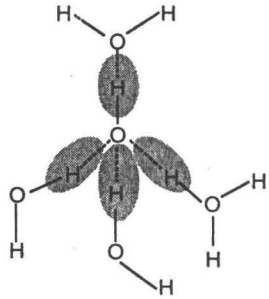
Fig. 3.1. Tetrahedral structure of water.



When two molecules of water are in close proximity, electrostatic attraction occurs between the partial negative charge on the oxygen atom of one water molecule and a partial positive charge on a hydrogen atom of its neighbour. This is accompanied by a redistribution of the electronic charges in both water molecules which enhances the attraction. This form of electrostatic attraction, called hydrogen bond, is not unique to water and has an important role in determining the structures of macromolecules. The arrangement of the electrons around the oxygen atom potentially allows the binding of four adjacent water molecules to any water molecule.

In liquid water, not all water molecules are fully hydrogen bonded; an average of 3.4 hydrogen bonds per molecule has been estimated. These bonds are constantly being formed and broken. The interlinking of the water molecules by hydrogen bonds is responsible for the internal cohesion of water resulting in its physical properties.

The high boiling and melting points of H_2O and its high heat of vaporization are the result of an interaction between adjacent water molecules known as hydrogen bonding.



The energy necessary to disrupt even the most stable hydrogen bonds (4–10 kcal/mole) is much less than that required to break most covalent bonds. In an aqueous solution, hydrogen

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bonds are broken and formed readily. The cumulative effect of hydrogen bonding in water is a major factor in explaining many of the unusual properties of H_2O. Thus, the extra energy required to boil water and melt ice may be attributed largely to extensive hydrogen bonding.

Hydrogen bonds play significant roles in biochemistry because they can be formed in large numbers. Multiple hydrogen bonds confer significant structure not only upon water but also upon other dipolar molecules as diverse as alcohols, DNA, and proteins. Fig. 3.2 illustrates hydrogen bonds formed between representative biochemicals.

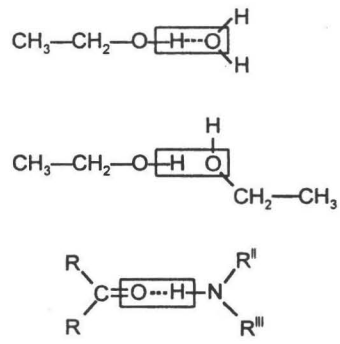

Fig. 3.2. Formation of hydrogen bonds between an alcohol and water, between 2 molecules of ethanol, and between the peptide carbonyl oxygen and the hydrogen on the peptide nitrogen of an adjacent peptide.

Hydrogen bonds are not restricted to water molecules. The hydrogens of nitrogen atoms can also participate in hydrogen bonding. This topic will be considered again in connection with the 3-dimensional structure of proteins and with base pairing in DNA.

3.2 Role of Water in Biological Systems

The unusual properties of water make it an ideal medium for living organisms. The specific heat capacity of H_2O—the number of calories required to raise the temperature of 1 g of water from 15 to 16°C—is 1.0 and is unusually high among several of the hydrogen bonding solvents considered in Table 3.1. Only a few solvents, such as liquid ammonia, have greater heat capacities than water. The greater the specific heat of a substance, the less the change in temperature that results when a given amount of heat is absorbed by that substance. Thus, H_2O is well-designed for keeping the temperature of a living organism relatively constant.

The heat of vaporization of water, as already mentioned, is unusually high. Expressed as the specific heat of vaporization (calories absorbed per gram vaporized), the value for water is 540 at its boiling point and even greater at lower temperatures. This high value is very useful in helping the living organism keep its temperature constant, since a large amount of heat can be dissipated by vaporization of H_2O.

The high heat of fusion of water (Table 3.1) is also of significance in stabilizing the biological environment. Although cellular water rarely freezes in higher living forms, the heat released by H_2O on freezing is a major factor in slowing the temperature drop of a body of water during the winter. Thus, a gram of H_2O must give up 80 times as much heat in freezing at 0°C as it does in being lowered from 1 to 0°C just before freezing.

One final example of a property of H_2O that is of biological significance must be cited : H_2O expands on solidifying, and ice is less dense than water. Only a few other substances expand on freezing. The importance of this property for biology has long been recognized. If ice were heavier than liquid water, it would sink to the bottom of its container on freezing. This would mean that oceans, lakes, and streams would freeze from the bottom to the top and, once frozen, would be extremely difficult to melt. Such a situation would obviously be incompatible with those bodies of water serving as the habitat of many living forms, as they do. As it is, however, the warmer, liquid water falls to the bottom of any lake and the ice floats on top where heat from the external environment can reach and melt it.

Additional properties of water such as high surface tension and a high dielectric constant have significance in biology.

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Table 3.1
SOME PHYSICAL PROPERTIES OF WATER AND OTHER COMPOUNDS

Substance	*Melting Point (°C)*	*Boiling Point (°C)*	*Heat of Vaporization (cal/g)*	*Heat Capacity (cal/g)*	*Heat of Fusion (cal/g)*
Water	0	100	540	1.00	80
Ethanol	– 114	78	204	0.58	25
Methanol	– 98	65	263	0.60	22
NH_3	– 78	– 33	327	1.12	84
H_2S	– 83	– 60	132	—	17
HF	– 92	19	360	—	55

3.3 Ionisation of Water

The electron of the hydrogen atom is attracted to the nucleus of the oxygen atom and so there is a tendency for the hydrogen nucleus (a proton) to dissociate from this water molecule. This proton will be attracted to the oxygen of another water molecule to "which it is hydrogen bonded forming a hydronium ion (H_3O^+) and a hydroxyl ion. The hydronium ion is usually considered to be a hydrated proton or hydrogen ion and is therefore designated by H^+.

Any reversible dissociation can be expressed in accordance with the Law of Mass Action which states 'at equilibrium, the mathematical product of the concentrations of the substances formed by a chemical reaction divided by the product of the concentrations of the reactants in that reaction is equal to a constant.' This constant is called the temperature-dependent equilibrium constant, K_{eq}. The equilibrium of a system is achieved when the rate of the reverse reaction equals the rate of the forward reaction. The dissociation of water may be written as :

$$H_2O \rightleftharpoons H^+ + OH^-$$

and

$$K_{eq} = \frac{[H^+[OH^-]}{[H_2O]}$$

where [ ] denotes concentration. An accurate experimental value for this K_{eq} has been obtained at 25°C (298 K) by electrical conductivity measurements :

$$K_{eq} = 1.8 \times 10^{-16} \text{ mol dm}^{-3}$$

Therefore, in pure water very little dissociation occurs so that the number of undissociated water molecules is very large and their concentration is essentially unchanged.

The above equation can also be written as :

$$K_{eq}\,[H_2O] = [H^+]\,[OH^-]$$

in which K_{eq} $[H_2O]$ is a new constant, called K_w, the ionic product of water since it is equal to the mathematical product of the concentrations of the ions formed by the dissociation of water. The value of K_w is 1×10^{-14} mol dm^{-3} from which the concentration of H^+ ions in pure water at 25°C may be calculated as 1.0×10^{-7} mol dm^{-3}.

3.4 Theories of Acids and Bases

There are three theories, named after their proposers, employed to explain the behaviour of the substances called acids or bases.

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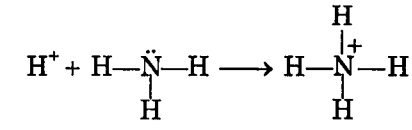
1. Arrhenius acids and bases in which an acid may be defined as a substance which dissociates in water to yield H^+ ions whilst a base may be defined by the potential to dissociate in water yielding OH^- ions.
2. Bronsted and Lowry acids and bases in which an acid is defined as any substance that can donate proton (H^+), *i.e.,* a proton donor. Conversely, a base is defined as any substance that can accept a proton, *i.e.,* a proton acceptor.
3. Lewis acids and bases in which a Lewis acid is defined as any substance which acts as an electron pair acceptor, conversely, a Lewis base is any substance which acts as an electron pair donor.

The Lewis definitions are consistent with the Bronsted and Lowry view because a proton can be considered to be an electron pair acceptor and a substance which accepts a proton as an electron pair donor, *e.g.,*



acid ammonia ammonium ion

Because of the aqueous environment of most biochemical reactions, the influence of ionization of the activity of biological compounds and the establishment of the pH scale, it is usual for the biochemist to consider acid and base behaviour in terms of the Bronsted and Lowry concept. According to the Bronsted-Lowry theory, an acid, HA, dissociates :

$$HA \rightleftharpoons H^+ + A^-$$

The dissociation is reversible and so A^- may interact with H^+ to re-from the acid. A^- is therefore acting as a proton acceptor and is, by definition, a base. The relationship of A^- to HA is denoted by the following terminology : HA and A^- are a conjugate acid-base pair, HA is the parent acid and A^- is the conjugate base of that acid.

3.5 Relative Strengths of Acids and Bases

Acids and bases can be identified as monoprotic or polyprotic depending upon the numbers of dissociable protons the acid contains or the number of protons with which the base can combine. Irrespective of whether a substance is monoprotic or polytic, the strength of an acid or base refers to the efficiency with which the substance demonstrates the properties of an acid or a base. For an acid, this means the ease with which it donates protons; for a base, this means the ease with which it accepts protons. These properties of a substance will be affected by its environment; it is easier for an acid to donate protons in a proton accepting medium. In biological systems, non-membranous environments are essentially aqueous.

With respect to strength, there are two general classes, strong and weak. Strong acid and bases are ones which are almost completely dissociated in dilute aqueous media. Weak acids and bases are ones which are only partially dissociated in dilute aqueous media. The strength of an acid or a base is most frequently determined by the dissociation constant, K. If B is a strong base, it will have a high affinity for H^+ and hence BH^+ is a weak acid. Conversely, if B is a weak base, it will have a low affinity for H^+ and so BH^+ is a strong conjugate acid. For this reason, the strength of a base is frequently expressed in terms of the acid dissociation constant of its conjugate acid, BH^+, *i.e.,*

$$K_a = \frac{[B]\,[H^+]}{[BH^+]}$$

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Therefore the strength of an acid is given by K_a and the strength of a base by K_b or K_a of its conjugate acid.

Table 3.2
THE STRENGTHS OF SOME MONOPROTIC WEAK ACIDS AND BASES

Substance	*Formula*	K_a	pK_a	K_b	pK_b	*Acidity or Basicity*
Formic acid	HCOOH	1.77×10^{-4}	3.75	—	—	↑
Acetic acid	CH_3COOH	1.76×10^{-5}	4.75	—	—	Increasing
Trimethylacetic acid	$(CH_3)_3C.COOH$	9.4×10^{-6}	5.03	—	—	acidity ⋮
Ammonia	NH_3	5.62×10^{-10}	9.25	1.77×10^{-5}	4.75	Increasing
Trimethylamine	$(CH_3)_3N$	1.55×10^{-10}	9.81	8.32×10^{-5}	4.19	basicity
Dimethylamine	$(CH_3)_2NH$	1.85×10^{-11}	10.73	5.25×10^{-4}	3.28	↓

For weak acids and bases, there is a gradation of strength, *i.e.*, some are weaker than others as shown by K_a (or pK_a) values in Table 3.2. Biochemical interest in these substances lies in their potential impact on biomolecules.

Many biochemicals prossess functional groups that are weak acids or bases. One or more of these functional groups—carboxyl groups, amino groups, or the secondary phosphate dissociation of phosphate esters—are present in all proteins and nucleic acids, most coenzymes, and most intermediary metabolites. The dissociation behaviour (protonic equilibria) of weakly acidic and weakly basic functional groups is therefore fundamental to understanding the influence of intracellular pH on the structure and biochemical activity of these compounds. Their separation and identification in research and clinical laboratories is also facilitated by knowledge of the dissociation behaviour of their functional groups.

3.6 pH Scale

Hydrogen ion concentration values are complex figures to write and use in calculations since they contain negative powers of 10. In 1909, Sorensen introduced the term pH. The pH expresses $[H^+]$ of an aqueous solution as a logarithmic function. The pH of a solution equals the negative of the logarithm to the $base_{10}$ of its hydrogen ion concentration, *i.e.*, $pH = -\log_{10} [H^+]$. pH offers a convenient mechanism of expressing a wide range of $[H^+]$ in small positive numbers. The letter, p, is used to denote the negative logarithm to the $base_{10}$ of.

The pH scale from 0 to 14 covers all the hydrogen ion concentration. Measurements of pH can be easily performed using a pH-meter.

Table 3.3
THE pH SCALE

$[H^+]$ ($mol\ dm^{-3}$)	*pH*	$[OH]$ ($mole\ dm^{-3}$)	*pOH*	*Acidity or Basicity*
1.0 (10^0)	0	10^{-14}	14	
0.1 (10^{-1})	1	10^{-13}	13	
10^{-4}	4	10^{-10}	10	Increasing ↑ acidity
10^{-7}	7	10^{-7}	7	Neutral
10^{-10}	10	10^{-4}	4	Increasing ↓ basicity
10^{-13}	13	10^{1}	1	
10^{-14}	14	10^{0}	0	

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Example 3.1. Calculate the pH of a solution in which the hydrogen ion concentration is 4.2×10^{-4} mol dm^{-3}.

$$[H^+] = 4.2 \times 10^{-4} \text{ mol dm}^{-3} \text{ and}$$
$$pH = -\log (4.2 \times 10^{-4})$$

Take log by pocket calculator :

$$pH = -(-3.38)$$
$$pH = 3.38$$

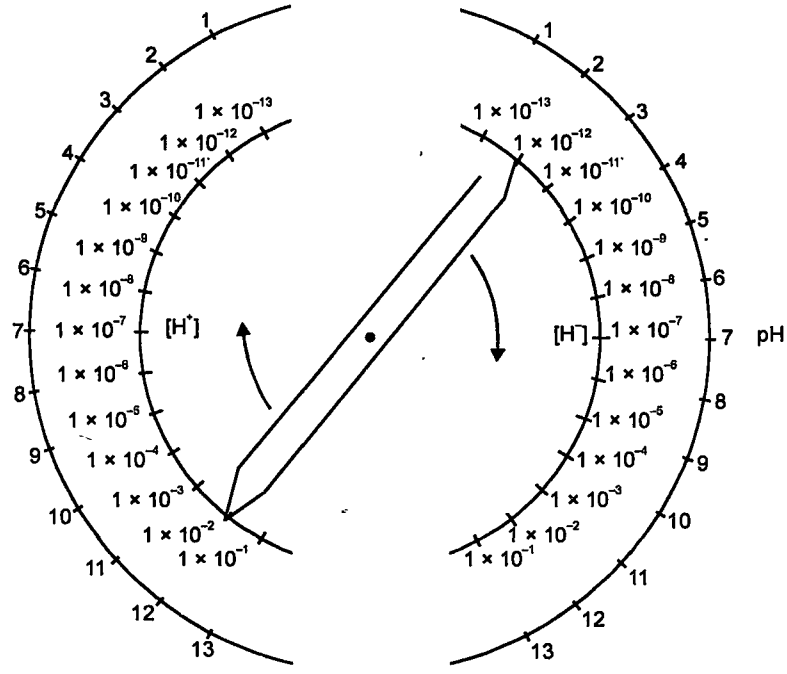

Fig. 3.3. $[H^+]$ versus $[OH^-]$ increases, $[OH^-]$ decreases; as $[H^+]$ decreases, $[OH^-]$ increases.

The pH is normally quoted to two decimal places and so the logarithm used is to two figures.

Example 3.2. Calculate the hydrogen ion concentration in a solution of pH 8.32.

$$pH = 8.32; \quad pH = -\log [H^+]$$
$$8.32 = -\log [H^+]$$
$$\log [H^+] = -8.32$$

Take antilog : $[H^+] = 4.79 \times 10^{-9}$ mol dm^{-3}

i.e., the $[H^+]$ in a solution of

$$\text{pH } 8.32 = 4.79 \times 10^{-9} \text{ mol dm}^{-3}$$

To meet normal metabolic demands, the human body required an enormous amount of oxygen on a continuous basis. The solubility of oxygen in blood would be very slight if not for the presence of the oxygen carrier, hemoglobin. Hemoglobin functions as a reversible oxygen carrier, picking up oxygen in the lungs and releasing in it tissues for use in cellular metabolism. The maintenance of a relative constancy of 7.4 of blood pH is vital for oxygen transport.

Inhaled oxygen from the lungs diffuses into the capillaries due to a difference in oxygen concentrations between the lungs and capillaries. Upon entering a capillary, oxygen diffuses into

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the red blood cells, where it combines with hemoglobin (HHb) to form oxyhemoglobin ($HHbO_2$), Combination with oxygen promotes the loss of a hydrogen ion from hemoglobin. The oxygenation reactions may be summarized as

$$O_2 + HHb \rightleftharpoons HHbO_2 \rightleftharpoons H^+ + HbO_2^- \quad ...(1)$$

Recalling Le Chatelier's principle, high oxygen concentration in the lungs favours the forward reaction, oxyhemoglobin formation. Additionally, bicarbonate ions in red blood cells remove hydrogen ions produced by Reaction (1) by combining with them to form carbonic acid, H_2CO_3. Thus, oxyhemoglobin formation and dissociation to HbO_2 are favoured by the high oxygen concentration and the buffering action of bicarbonate ions.

$$H_2O + CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \quad ...(2)$$

What does release of hydrogen by cells do to the equilibria in Equation (1) ? It places a stress on the equilibria; HbO_2^- ion combines with hydrogen ion to form $HHbO_2^-$, which splits into oxygen and hemoglobin. The oxygen diffuses into the cells for use in cellular metabolism. Thus, oxygen transport is a pH– controlled process; deoxygenation into active cells requires H^+ ions.

As hydrogen ions are removed by combination with HbO_2^-, bicarbonate concentration in red blood cells increases (Le Chatelier's principle again). When this occurs, bicarbonate ions diffuse out of the red blood cell into the plasma. To balance the loss of negative ions by the red blood cell, chloride ions migrate from the plasma into the cell, a process called the *chloride shift.*

3.7 Acidosis and Alkalosis

Altered carbonic acid and bicarbonate levels in the blood, if uncompensated, change the blood's pH. By decreasing the amount of breathing (hypoventilation), insufficient carbon dioxide is released. *The increased level of carbon dioxide in the blood leads to increased carbonic acid and hydrogen ion levels and respiratory acidosis results.* Fainting can occur from mild acidosis; coma can be the result of severe acidosis. Alternatively, by hyperventilating (overbreathing), an excessive amount of carbon dioxide is expelled. Equation (2) indicates that excessive loss of carbon dioxide shifts the equilibrium to compensate for CO_2 loss. Hydrogen ions and bicarbonate ions combine to form carbonic acid, which decomposes to water and carbon dioxide. *The reduction in hydrogen ion concentration leads to respiratory alkalosis.*

Acidosis and alkalosis can also occur due to metabolism. Normal carbohydrate metabolism results in the formation of acidic by-products, which can be termed *metabolic acids.* Lactic, citric, and pyruvic acids are examples of metabolic acids. Their concentration is ordinarily such that normal body buffer action prevents continuing acidosis. In diabetes mellitus, *abnormal* carbohydrate metabolism leads to acidosis. Lack of insulin secretion by the pancreas prevents glucose from being metabolized. In its continuing need for energy, the body instead metabolized stored fats. Two of the products of fat metabolism are acids. Their dissociation increases the hydrogen ion concentration in extracellular fluids causing metabolic acidosis. If untreated, severe acidosis leads to coma and even death.

3.8 Buffers

A *buffer solution* is a solution whose pH remains essentially constant despite the addition of a *small* amount of acid or base. Buffers are enormously important to chemical and biological systems. The pH of human body fluids varies greatly, depending on the location. For example, the pH of blood plasma is about 7.4, whereas that of gastric juice, a fluid produced by glands in the mucosa membrane lining the stomach, is about 1. Yet these pHs must be maintained by buffers for the proper functioning of enzymes, the balance of osmotic pressure, and so on. Table 3.3 lists the pH of a number of fluids.

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Table 3.4
pH VALUES OF VARIOUS MATERIALS

Material	*pH Value*
Body fluids and tissues	
Blood serum	7.35—7.45
Cerebrospinal fluid	7.35—7.45
Aqueous humor of eye	7.4
Saliva	6.35—6.85
Pure gastric juice	About 0.9
Pancreatic juice	7.5—8.0
Intestinal juice	7.0—8.0
Hepatic duct bile	7.4—8.5
Gallbladder bile	5.4—6.9
Urine	4.8—7.5
Feces	7.0—7.5
Tears	7.4
Milk	6.6—6.9
Skin (intracellular, various layers)	6.2—7.5
Liver (intracellular)	
Kupffer cells	6.4—6.5
Peripheral cells	7.1—7.4
Current cells	6.7—6.9
Miscellaneous	
Distilled water, exposed to air from which it absorbs some CO_2	About 5.5
Sea water	8.0
Vinegar	3.0
Orange juice	2.6—4.4
Grapefruit juice	3.2
Tomatoes (ripe)	4.3
Egg white (fresh)	8.0

Henderson-Hasselbalch Equation

Henderson and Hasselbalch have rearranged the law of mass equation, as it applies to the ionisation of weak acids into a useful expression known as the Henderson-Hasselbalch equation. Consider the ionisation of a generalised weak acid, HA.

$$HA \rightleftharpoons H^+ + A^- \qquad ...(i)$$

On applying law of mass action to the above equation, we get

$$K_{ion} = K_a = \frac{[H^+][A^-]}{[HA]}$$

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or $$[H^+] = \frac{K_a[HA]}{[A^-]}$$

On taking logarithms, we find

$$\log [H^+] = \log K_a + \log \frac{[HA]}{[A^-]}$$

and multiplying by – 1, we get

$$-\log H^+ = -\log K_a - \log \frac{[HA]}{[A^-]}$$

If $-\log H^+$ is defined as pH, $-\log K_a$ is defined as pK_a and $\log [A^-]/[HA]$ is substituted for $-\log [HA]/[A^-]$, we obtain

$$pH = pK_a + \log \frac{[A^-]}{[HA]} \quad ...(ii)$$

If $[A^-] = [HA]$, then

$$pH = pK_a + \log [1]$$

or $$pH = pK_a \quad [\because \log 1 = 0]$$

The Henderson-Hasselbalch equation can be written in a more general expression in which we replace $[A^-]$ with the term "conjugate base" and [HA] with conjugate acid.

$$pH = pK_a = \log \frac{[\text{conjugate base}]}{[\text{conjugate acid}]} \quad ...(iii)$$

This expression may then be applied not only to weak acids such as acetic acid but also to the ionisation of ammonium ions and those substituted amino groups found in amino acids.

Henderson-Hasselbalch equation is quite useful in buffer and acid-base titration calculations, as we shall see in the following example.

Example 3.3. Calculate the pH of the 1.0 M CH_3COOH/1.0 M CH_3COONa buffer system. What is the pH after the addition of 0.10 mol of HCl to 1 liter of this buffer?

Answer : In the buffer we have

$$CH_3COONa \longrightarrow CH_3COO^- + Na^+$$

and

$$CH_3COOH \rightleftharpoons CH_3COO^- + H^+$$

Since acetic acid is a weak acid, as a good approximation we can write

$$[CH_3COOH] \simeq 1.0 \text{ M}$$

and

$$[CH_3COO^-] \simeq 1.0 \text{ M}$$

From Eq. (*iii*), we have

$$pH = 4.76 + \log \frac{1.0}{1.0} = 4.76$$

or

$$[H^+] = 1.75 \times 10^{-5} \text{ M}$$

After the addition of 0.10 mol of HCl, the following reaction takes place :

CH_3COO^-	+	H^+	$\longrightarrow$	CH_3COOH
0.10 M		0.10 M		0.10 M

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Again we have neglected the small contribution to the total hydrogen-ion concentration which results from the ionization of acetic acid. The new concentrations are

$$[CH_3COO^-] = 1.0 - 0.10 = 0.90 \text{ M}$$

and

$$[CH_3COOH] = 1.0 + 0.1 = 1.10 \text{ M}$$

Hence the pH of the buffer is

$$\text{pH} = 4.76 + \log \frac{0.90}{1.10} = 4.67$$

or

$$[H^+] = 2.13 \times 10^{-5} \text{ M}$$

Thus there is a change of about 0.1 unit on the pH scale, equivalent to an increase of $[H^+]$ by a factor of $2.13 \times 10^{-5}/1.75\ 5\ 10^{-5}$, or 1.22.

To see how effective this buffer is against the acid, let us compare the pH change when 0.10 mol of HCl is added to 1 liter of water. The pH decreases from 7 to 1, amounting to a millionfold increase in $[H^+]$!

Often it is necessary to know the effectiveness of a buffer on a quantitative basis. To do so, we employ the term *buffer capacity* (β), first introduced by van Slyke in 1922. Buffer capacity is defined as the amount of acid or base that must be added to the buffer to produce a unit change of pH. Hence,

$$\beta = \frac{d[B]}{d\text{pH}}$$

The buffer capacity always has a positive value, however, since addition of base increases the pH and addition of acid decreases the pH. Thus $d[B]$ and dpH always have the same signs. The value of β depends not only on the nature of the buffer, but also on the pH, which is determined by the relative concentrations of the acid and its conjugate base. Figure 3.4 shows plots of buffer capacity versus pH for the CH_3COOH—CH_3COONa system. We see that the buffer functions best around its pK_a value of 4.76. This is not surprising, for according to Eq. (1), $\text{pH} = pK_a$ when $[HA] = [A^-]$, and there are equal amounts of acid and conjugate base to react with the added base or acid.

All that has been said so far applied equally well to the buffer system of a weak base (B) and its conjugate acid (BH^+), for which the reader should be able to derive the following Henderson-Hasselbalch equation :

$$\text{pH} = pK_a + \log \frac{[B]}{[BH^+]}$$

The criteria for buffers suitable for use in biological research may be summarised as follows. They should :

(*i*) possess adequate buffer capacity in the required pH range;

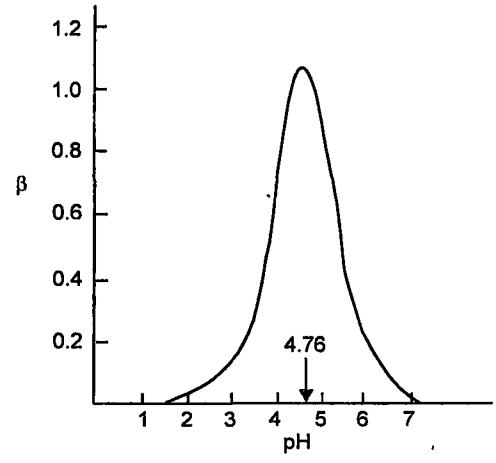

Fig. 3.4. Buffer capacity of the 1 M CH_3COOH/ 1 M CH_3COONa buffer system. The maximum of the peak occurs at pH = 4.76, which is also equal to pK_a.

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(*ii*) be available in a high degree of purity;
(*iii*) be very water soluble and impermeable to biological membranes;
(*iv*) be enzymatically and hydrolytically stable;
(*v*) possess a pH which is minimally influenced by their concentration, temperature and ionic composition or salt effect of the medium;
(*vi*) not be toxic or biological inhibitors;
(*vii*) only form complexes with cations that are soluble;
(*viii*) not absorb light in the visible or ultraviolet regions.

Needless to say, not all buffers which are commonly used meet all these criteria. Thus, phosphates tend to precipitate polyvalent cations and are often metabolites or inhibitors in many systems and Tris is often toxic or may have inhibitory effects. However, a number of zwitterionic buffers of the HEPES and PIPES type fulfil the requirements and are often used in tissue culture media containing sodium bicarbonate or phosphate solutions as nutrients. They suffer from the disadvantage that they interfere with Lowry protein determinations.

Physiologically one of the most important groups of buffers is the proteins. By virtue of their large numbers of weak acidic and basic groups in the amino acid side chains, proteins have a very high buffer capacity. Hemoglobin is mainly responsible for the buffer capacity of blood.

To obtain buffer solutions covering an extended pH range, but which are derived from the same ions, mixtures of different systems may be used. Thus the McIlvaine buffers cover the pH range 2.2 to 8.0 and are prepared from citric acid and disodium hydrogen phosphate.

A number of common buffer solutions are given in Table 3.5.

Table 3.5
SOME COMMON BUFFER SOLUTIONS

Buffer	*pH Range*
KH phthalate/phthalic acid	2.1 – 4.1%
CH_3COONa/CH_3COOH	3.8 – 5.8
KNa phthalate/KH phthalate	4.4 – 6.4
Na_2HPO_4/KH_2PO_4	6.2 – 8.2
Tris (hydroxymethyl) aminomethane/HCl	7.1 – 9.1
Naborate/boric acid	8.1 – 10.1
$Na_2CO_3/NaHCO_3$	9.3 – 11.3
Na_3PO_4/Na_2HPO_4	11.3 – 13.3

Table 3.6 includes some important buffering systems of body fluids which help in maintaining pH. A certain amount of many of these is usually present in the body and cellular fluids, and so the maintenance of a constant pH is dependent on a complex system.

Table 3.6
BODY FLUIDS AND THEIR PRINCIPAL BUFFERS

Body fluids	*Principal buffers*
Extracellular fluids	Bicarbonate buffer Protein buffer
Intracellular fluids	Phosphate buffer Protein
Erythrocytes	Hemoglobin buffer

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Some Important Biological Buffering Systems

Some of these are described as follows :

I. The phosphate buffer system : This system acts in the cytoplasm of all cells. It consists of $H_2PO_4^-$ as proton donor while HPO_4^{2-} as proton acceptor :

$$H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-}$$

The working of phosphate buffer system is exactly like the acetate buffer system, except for the pH range in which it functions. The phosphate buffer system is maximally effective at a pH close to its pK_a of 6.86 and thus tends to resist pH changes in the range between 6.4 and 7.4. It therefore, finds use in providing buffering power in intracellular fluids.

The concentration of phosphate buffer in the blood plasma has been found to be about 8% of that of the bicarbonate buffer. Therefore its buffering capacity would be much lower than bicarbonate in the plasma. The concentration of phosphate buffer is much higher in intracellular fluid than in extracellular fluids. The pH of intracellular fluids (6.0–6.9) is nearer to the pK_a of the phosphate buffer. Hence, the buffering capacity of the phosphate buffer gets highly elevated inside the cells and the phosphate also tends to be effective in the urine inside the renal distal tubules and collecting ducts.

If the ratio of $[HPO_4^{2-}]/[H_2PO_4^-]$ tends to get changed by the formation of more $H_2PO_4^-$, there occurs the renal elimination of $H_2PO_4^-$ for which the ratio ultimately does not get altered.

II. The bicarbonate buffer system : It is considered to be the main extracellular buffer system which (also) offers a means for the necessary removal of the CO_2 formed by tissue metabolism. The bicarbonate buffer system has been the main buffer in blood plasma and is consisting of carbonic acid as proton donor while bicarbonate as proton acceptor :

$$H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

The equilibrium constant of this system is :

$$K_1 = \frac{[H^+]\,[HCO_3^-]}{[H_2CO_3]}$$

This system functions as a buffer in the same way as other conjugate acid-base pairs do. However it is unique in that one of its components, carbonic acid gets produced from dissolved (*d*) carbon dioxide and water, according to the reversible reaction :

$$CO_2(d) + H_2O \rightleftharpoons H_2CO_3$$

The above equilibrium has an equilibrium constant given by the following expression :

$$K_2 = \frac{[H_2CO_3]}{[CO_2(d)]\,[H_2O]}$$

But carbon dioxide is a gas under natural conditions. Therefore, the concentration of dissolved CO_2 is the result of equilibration with CO_2 of the gas phase (*g*) :

$$CO_2\,(g) \rightleftharpoons CO_2(d)$$

This process is having an equilibrium constant given by :

$$K_3 = \frac{[CO_2(d)]}{[CO_2(g)]}$$

Thus, the pH of a bicarbonate buffer system tends to depend on the concentration of H_2CO_3 and HCO_3^-, the proton donor and acceptor components. The concentration of H_2CO_3, in turn,

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tends to depend on the concentration of dissolved CO_2, which, in turn, tends to depend on the concentration or partial pressure of CO_2 in the gas phase.

With respect to the bicarbonate system, a $[HCO_3^-]/[H_2CO_3]$ ratio of 20 to 1 is essential for the pH of blood plasma to remain at 7.40. The concentration of dissolved CO_2 has been also included in the $[H_2CO_3]$ value, *i.e.*,

$$[H_2CO_3] = [H_2CO_3] + [CO_2 \text{ (dissolved)}]$$

When there occurs a change in the ratio in favour of H_2CO_3, *acidosis* is said to take place. This change can occur due to a decrease in $[HCO_3^-]$ or due to an increase in $[H_2CO_3]$. Most common forms of acidosis happen to be metabolic or respiratory. *Metabolic acidosis* is attributed to a decrease in $[HCO_3^-]$ and takes place, for example, in uncontrolled diabetes with ketosis or because of starvation. *Respiratory acidosis* is said to take place if there occurs an obstruction to respiration (euphysema, asthma or pneumonia) or depression of respiration (toxic doses of morphine or other respiratory depressants). If acidosis is not treated promptly, the patient may go into a comma.

Alkalosis is said to occur if $[HCO_3^-]$ gets favoured in the bicarbonate/carbonic acid ratio. *Metabolic alkalosis* is said to occur if the HCO_3^- fraction increases with little or no concomitant change in H_2CO_3. Severe vomiting (loss of H^+ as HCl) or ingestion of excessive amounts of sodium bicarbonate (bicarbonate of soda) are known to produce this condition. *Respiratory alkalosis* is said to be induced by hyperventilation since an excessive removal of CO_2 from the blood gives rise to a decrease in $[H_2CO_3]$. Hyperventilation can produce anxiety, hysteria, prolonged hot baths or lack of O_2 at high altitudes. Alkalosis can produce convulsive seizures in children and tetany, hysteria, prolonged hot baths or lack of O_2 as high altitudes. Alkalosis is responsible for convulsive seizures in children and tetany in adults (characterized by sharp flexion of the wrist and ankle joints, muscle switching, and cramps).

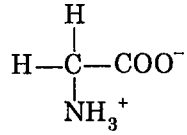
The pH of blood is maintained at 7.4 provided the buffer ratio $[HCO_3^-]/[H_2CO_3]$ remains at 20. If the bicarbonate neutralizes any acid or base, there may occur the change of buffer ratio and the blood pH value. But the buffer ratio is maintained by the respiratory elimination of H_2CO_3 as CO_2 or the urinary elimination of HCO_3^-.

As cells are having much lower amounts of HCO_3^-, the importance of bicarbonate buffer inside the cell tends to be negligible.

III. The protein buffer systems : The protein buffers are considered to be very important in the plasma and the intracellular fluids but their concentration tends to be very low in cerebrospinal fluid, lymph and interstitial fluids. The proteins exist as anions serving as conjugate bases (Pr^-) at the blood pH 7.4 and produce conjugate acids (HPr) accepting H^+. They possess the capacity to buffer some H_2CO_3 in the blood.

$$H_2CO_3 + Pr^- \rightleftharpoons HCO_3^- + HPr$$

IV. The amino acids buffer system : This system also works in humans. Amino acids in their molecules have both an acidic (– COOH) and a basic (– NH_2) group. They exist in the form of a neutral zwitterion in which a hydrogen atom can go between the carboxyl and amino groups. The glycine may, thus, be represented as :



On the addition or subtraction of a hydrogen ion to or from the zwitterion, either the cation or anion form will be formed.

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$$\underset{\text{Cation form}}{{}^{+}H_3N{-}CH_2{-}COOH} \xleftarrow{+H^+} \underset{\text{Zwitterion}}{{}^{+}H_3N{-}CH_2{-}COO^-} \rightleftharpoons \underset{\text{Anion form}}{H_2N{-}CH_2{-}COO^-} + H^+$$

Thus, if OH^- ions are added to the solution of amino acid, they accept H^+ from it to form water, and the anion is formed. If H^+ ions are added, they are accepted by the zwitterion to form the cation form. In practice, if NaOH is added, the salt $H_2N{-}CH_2{-}COONa$ would be formed and the addition of HCl would give rise to the formation of amino acid hydrochloride, $ClH{-}H_2N{-}CH_2{-}COOH$, but these substances would ionize in solution to some extent to yield their corresponding ions. Hemoglobin and plasma proteins act as buffers in a similar manner.

Amino acids tend to differ in the degree to which they will be able to produce the cation or anion form. In other words it means that a solution of an amino acid is not neutral but is either predominantly acidic or basic, depending on which form is present in greater quantity. Because of this reason, different amino acids may find use as buffers for different pH values, and a mixture of them is having a wide buffer range.

V. The hemoglobin buffer systems : These buffer systems take part in buffering CO_2 inside erythrocytes. The buffering capacity of hemoglobin has been found to depend on its oxygenation and deoxygenation. Inside the erythrocytes, CO_2 combines with H_2O to yield carbonic acid (H_2CO_3) under the action of carbonic anhydrase. At the blood pH 7.4, H_2CO_3 gets dissociated into H^+ and HCO_3^- and requires immediate buffering. Oxyhemoglobin (HbO_2^-), on the other side, tends to lose O_2 to form deoxyhemoglobin (Hb^-) which is not dissociated (HHb) by accepting H^+ from the ionization of H_2CO_3. Hence, Hb^- buffers H_2CO_3 in erythrocytes :

$$HbO_2^- \rightleftharpoons Hb^- + O_2$$

$$Hb^- + H_2CO_3 \rightleftharpoons HHb + HCO_3^-$$

Some HCO_3^- tends to diffuse out into the plasma to maintain the balance between intracellular and plasma bicarbonates. This brings about influx of some Cl^- into erythrocytes along the electrical gradient formed by the HCO_3^- outflow (*chloride shift*).

$HHbO_2$, formed in lungs by oxygenation of HHb, immediately undergo ionisation into H^+ and HbO_2^-. The released hydrogen ions (H^+) get buffered by HCO_3^- inside erythrocyte to yield H_2CO_3 which gets dissociated into H_2O and CO_2 by carbonic anhydrase. CO_2 gets diffused out of erythrocytes and escapes in the alveolar air. Some HCO^-_3 return from the plasma to erythrocytes in exchange of Cl^- and get changed to CO_2.

$$HHb + O_2 \rightleftharpoons HHbO_2 \rightleftharpoons HbO_2^- + H^+$$

$$HCO_3^- + H^+ \rightleftharpoons H_2CO_3 \rightleftharpoons H_2O + CO_2$$

Use of Buffers in Maintaining the pH in Blood

The pH of most intracellular fluids varies between 6.8 and 7.8. Among the large number of buffer systems needed to maintain the proper pH are HCO_3^- H_2CO_3 and HPO_4^{2-}–$H_2PO_4^-$. It is known that, around pH 7.3 the predominant buffers are bicarbonate and carbonic acid and HPO_4^{2-} and $H_2PO_4^-$. They react with acids and bases as follows :

$$HA + HCO_3^- \rightleftharpoons A^- + H_2CO_3$$

$$B + H_2CO_3 \rightleftharpoons BH^+ + HCO_3^-$$

and

$$HA + HPO_4^{2-} \rightleftharpoons A^- + H_2PO_4^-$$

$$B + H_2PO_4^- \rightleftharpoons BH^+ + HPO_4^{2-}$$

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In an adult weighing 70 kg, it is estimated that about 0.1 mol of H^+ ions and 12 mol of CO_2 are produced everyday as a result of metabolism. The body has two mechanisms for handling the acid produced by metabolism to prevent a lowering of pH : buffering and excreting H^+. We shall concentrate first on the buffering action of blood. Blood consists essentially of two components : blood plasma, a complex solution containing many biochemically important compounds (carbohydrates, amino acids, proteins, enzymes, hormones, vitamins and inorganic ions), and erythrocytes or red blood cells. The blood of the average adult contains about 5 million erythrocytes per milliliter of blood. Hemoglobin, the "respiratory" protein, is present inside the erythrocyte. There are about 2×10^8 hemoglobin molecules per erythrocyte. Blood plasma is maintained at pH 7.4 largely by the HCO_3^-/H_2CO_3 and $HPO_4^{2-}/H_2PO_4^-$ buffers, and by various plasma proteins. Proteins are polyamino acids which can themselves act as buffers. A particularly important plasma protein, called albumin, contains 16 histidine residues per molecule. It is known that the imidazole group of the histidine molecule in proteins can combine with the buffer H^+. Equation (*iii*) can be used to estimate the relative concentrations of H_2CO_3 and HCO_3^- as follows. At pH 7.4 we write,

$$7.4 = 6.4 + \log \frac{[HCO_3^-]}{[H_2CO_3]}$$

or $[HCO_3^-]/[H_2CO_3] = 20$. In the normal person, the concentration of CO_2 is nearly as 1.2×10^{-3} M and HCO_3^- is about 0.024 M, giving a ratio of 24/1.2 or 20, as we calculated above. In the erythrocytes the buffers are HCO_3^-/H_2CO_3 and hemoglobins. (Hemoglobin molecules also possess histidine residues.) There the pH is about 7.25. Again from Eq. (1) the $[HCO_3^-]/[H_2CO_3]$ is found to be about 14. The membrane of the red blood cell is more permeable to anions such as HCO_3^-, OH^-, and Cl^- than to K^+ and Na^+ cations.

Oxyhemoglobin, formed by the combination of oxygen with hemoglobin in the lungs, is carried in the arterial blood to the capillary beds, where the oxygen is unloaded to the tissues via myoglobin. Both hemoglobin and oxyhemoglobin are weak acids, although the latter is considerably stronger than the former :

$$HHb \longrightarrow H^+ + Hb^- + pK_a = 8.2$$
$$HHbO_2 \longrightarrow H^+ + HbO_2^- \; pK_a = 6.95$$

where HHb and $HHbO_2$ represent "monoprotic" hemoglobin and oxyhemoglobin, respectively. This means that at pH = 7.25, about 65% of $HHbO_2$ is in the dissociated form, while only 10% of HHb is dissociated. The release of oxygen by $HHbO_2$ is strongly influenced by the presence of carbon dioxide. In metabolizing tissues, the partial pressure of CO_2, P_{CO_2}, is higher in the interstitial fluid (that is, fluid within the tissue space) than in the plasma. Thus it diffuses into the blood vessel and then into the erythrocytes. Here, most of the CO_2 is converted to H_2CO_3 by the *enzyme carbonic anhydrase :*

$$CO_2 + H_2O \longrightarrow H_2CO_3$$

The presence of H_2CO_3 lowers the pH, which has a direct effect on the release of oxygen. Oxygen may be released from either $HHbO_2$ or HbO_2^- as shown :

$$HHbO_2 \longrightarrow HHb + O_2$$
$$HHbO_2 \longrightarrow H^+ + HbO_2^-$$
$$HbO_2^- \longrightarrow Hb^- + O_2$$

As $HHbO_2$ releases oxygen more readily than HbO_2^-, a lowering in pH increases the concentration of $HHbO_2$ and promotes the first step. The conjugate base Hb^- of the weaker acid HHb has a greater tendency to react with H_2CO_3 as follows :

$$Hb^- + H_2CO_3 \longrightarrow HHb + HCO_3^-$$

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The bicarbonate ion formed passes through the membrane and is carried away in the plasma. This is the major mechanism for the elimination of CO_2. When the venous blood circulates back to the lungs, where P_{CO_2} is low and P_{O_2} is high, hemoglobin recombines with oxygen to form oxyhemoglobin :

$$HHb + O_2 \longrightarrow HHbO_2$$

The bicarbonate ions in blood plasma now diffuse into the erythrocyte to raise the pH, and we have

$$HHbO_2 + HCO_3^- \longrightarrow HbO_2^- + H_2CO_3$$

The H_2CO_3 is then converted to CO_2, catalyzed by carbonic anhydrase :

$$H_2CO_3 \longrightarrow CO_2 + H_2O$$

Because of the lower P_{CO_2} in the lungs, the CO_2 formed diffuses out of the erythrocyte and is then exhaled into the atmosphere.

What causes the bicarbonate ions formed in red blood cells to preferentially diffuse into plasma? The Donnan equilibrium, gives the answer to this question. The concentration of Hb^- and HbO_2^- is quite high in the erythrocytes; consequently there is an unequal distribution of the diffusible anions in the erythrocytes and in the plasma. Now, according to the Donnan equilibrium, the concentrations of the HCO_3^-, Cl^-, and OH^- ions are greater in the plasma than in the erythrocyte (assuming the proteins to be in the anion form). Further, for any given salt MX we can write

$$(\mu_{Mx})^C = (\mu_{Mx})^P$$

where C and P denote red blood cell and blood plasma. Following the same procedure as that used for the Donnan equilibrium, we arrive at the result

$$[M^+]_C [X^-]_C = [M^+]_P [X^-]_P$$

or

$$\frac{[M^+]_P}{[M^+]_C} = \frac{[X^-]_C}{[X^-]_P}$$

Thus for a given cation and varying the anions we can show that

$$\frac{[HCO_3^-]_C}{[HCO_3^-]_P} \frac{[Cl^-]_C}{[Cl^-]_P} \frac{[OH^-]_C}{[OH^-]_P}$$

At the capillary beds, the CO_2 diffuses into the red blood cell and is converted into H_2CO_3. The carbonic acid reacts with Hb^- and HbO_2^- to form bicarbonate ion, causing the ratio $[HCO_3^-]_C/[HCO_3^-]_P$ to increase. For proper balance of ionic concentrations, the bicarbonate ions diffuse into the plasma, while the chloride and hydroxide ions diffuse into the cell, maintaining electrical neutrality until the above equalities are restored. There is also a corresponding decrease in pH in the erythrocyte caused by the departure of HCO_3^- ions, but this is balanced by the flow of OH^- ions in the reverse direction. Since

$$[H^+]_C [OH^-]_C = [H^+]_P [OH^-]_P$$

or

$$\frac{[OH^-]_C}{[OH^-]_P} = \frac{[H^+]_P}{[H^+]_C}$$

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we see that the difference in pH is always maintained in the cell and in the plasma. Figure 3.5 Summarizes this discussion.

The phenomenon described above is sometimes called the bicarbonate chloride shift. In the lungs, the process is exactly reversed. There the bicarbonate ion reacts with oxygenated hemoglobin, causing the ratio $[HCO_3^-]_C/[HCO^{3-}]_P$ to decrease. The HCO_3^- ions diffuse from the plasma into the erythrocyte, while the Cl^- and OH^- ions diffuse in the opposite direction, until all the ratios of concentrations are again equal.

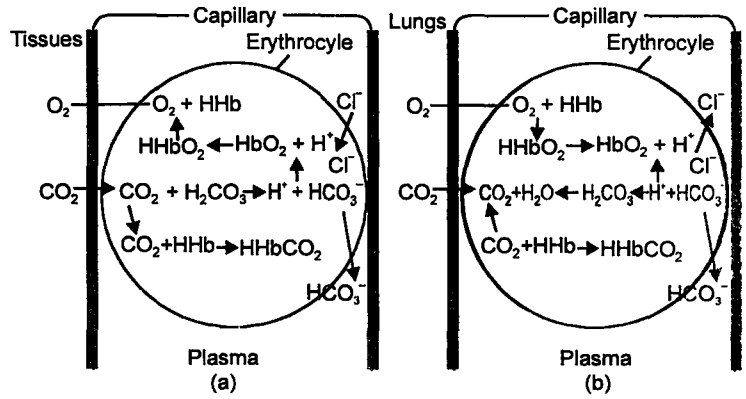

Fig. 3.5. Oxygen-carbon dioxide transport and release by blood. (*a*) In metabolizing tissues, the partial pressure of CO_2 is higher in the interstitial fluid (fluid in the tissues) than in the plasma. Thus it diffuses into the blood capillaries and then into erythrocytes. There it is converted to carbonic acid by the enzyme carbonic anhydrase (CA). The protons provided by the carbonic acid then combine with the oxyhemoglobin anions to form $HHbO_2$, which eventually dissociates into HHb and O_2. Because the partial pressure of O_2 is higher in the erythrocytes than in the interstitial fluid, oxygen molecules will diffuse out of the erythrocytes and then into the tissues. The bicarbonate ions also diffuse out of the erythrocytes and are carried by the plasma to the lungs. A small portion of the CO_2 also binds to hemoglobin to form carbamino hemoglobin. (*b*) In the lungs, the processes are exactly reversed.

The foregoing discussion outlines the efficient and fascinating ways our bodies make use of the buffering action. But buffering alone is not enough. The excretion of constantly forming H^+ ions also lays an important role in maintaining a normal blood pH. The kidneys can excrete H^+ ions and return HCO_3^- ions back to the blood, which can then combine with more H^+ ions. Normally, the pH of urine lies between 4.8 and 7.5. But from blood that has a pH of 7.4, the kidneys can produce a urine with a pH as low as 4.5. Two other mechanisms through which the kidneys can excrete H^+ ions are worth noting. The first is the excretion of anions of weak acids, particularly phosphoric acid. At pH 7.4, roughly one-third of the phosphate is present as $H_2PO_4^-$ and two-thirds as HPO_4^{2-}. In an acid urine, however, most of the phosphate will be excreted as $H_2PO_4^-$. The second mechanism involves the formation of NH_4^+ ions. Amino acids are degraded by the kidneys to form ammonia, which combines with H^+ ions as follows :

$$NH_3 + H^+ \longrightarrow NH_4^+$$

The ammonium ions are then excreted in the urine.

3.10 Donnan Effect

It describes the distribution of small diffusible ions on the two sides of a membrane, freely permeable to these ions but impermeable to macromolecular ions, in the presence of a macromolecular electrolyte on one side of the membrane. Suppose that a cell is separated into two components by a semipermeable membrane that allows the diffusion of water and small ions but not protein molecules. Let us consider the following three cases :

Case 1 : The protein solution is placed in the left compartment, water placed in the right compartment. We assume that the protein molecules are neutral species. Let the

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concentration of the protein solution be (mol liter^{-1}) so that the osmotic pressure of the solution is given by

$$\pi_1 = cRT$$

Thus from a measurement of the osmotic pressure we can readily determine the molar mass of the protein molecule.

Case 2 : In this case the protein is present as the anion of the sodium salt Na^+P^-, which we assume to be a strong electrolyte. Again, the protein solution of concentration c is placed in the left compartment and pure water in the right compartment. To maintain electrical neutrality, all of the Na^+ ions remain in the left compartment; the osmotic pressure of the solution now becomes

$$\pi_2 = (c + c)\,RT = 2cRT$$

Since $\pi_2 = 2\pi_1$, it follows that the molar mass determined in this case will only be half that of the true molar mass. It is known that $M_2 = c_2\,RT/\pi$, so that doubling π would decrease the value of M_2 by half. In practice the situation is actually much worse, since the protein ion may bear as many as 20 or 30 net negative (or positive) charges. In the early days of protein-molar-mass determination by osmotic pressure, disastrously poor results were obtained when the dissociation process was not recognized and efforts made to correct it.

Case 3 : We start with an arrangement similar to that discussed in Case 2 and then add NaCl (of concentration b in mol liter^{-1}) to the right compartment (Figure 3.6). At equilibrium, a certain amount, x (mole liter^{-1}), of Na^+ and Cl^- ions have diffused through the membrane from right to left, creating a final state shown in Figure 3.6*b*. Both sides of membrane must be electrically neutral: In each compartment the number of cations equal the number of anions. The condition of equilibrium allows us to equate the chemical potentials of NaCl in the two compartments as follows :

$$(\mu_{NaCl})^L = (\mu_{NaCl})^R$$

or

$$(\mu^0 + RT \ln a_\pm)^L_{NaCl} = (\mu^0 + RT \ln a_\pm)^R_{NaCl}$$

Since μ^0, the standard chemical potential, is the same on both sides, we obtain

$$(a_\pm)^L_{NaCl} = (a_\pm)^R_{NaCl}$$

We know that

$$(a_{Na^+} + a_{Cl^-})^L = (a_{Na^+} + a_{Cl^-})^R$$

If the solutions are dilute, the ionic activities may be replaced by the corresponding concentrations, that is, $a_{Na^+} = [Na^+]$ and $aCl^- = [Cl^-]$. Hence,

$$[(Na^+)\,(Cl^-)]^L = ([Na^+]\,[Cl^-])^R$$

or

$$(c + x)_x = (b - x)\,(b - x)$$

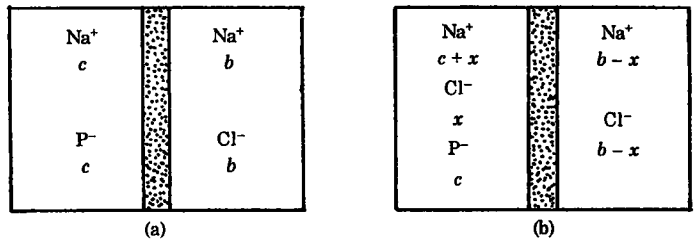

Fig. 3.6. Donnan equilibrium : (*a*) before diffusion has begun; (*b*) at equilibrium. The membrane separating the left and right compartments is permeable to all but the P^- ions.

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Table. 3.7

Initial Concentration		*Equilibrium Concentration*		
Left Compartment	*Right Compartment*	*Left Compartment*		
$c = [Na^+] = [P^-]$	$b = [Na^+] = [Cl^-]$	$(c - x) = [Na^+]$	$c = [P^-]$	$x = [Cl^-]$
0.1	0	0.1	0.1	0
0.1	0.01	0.1008	0.1	0.00083
0.1	0.1	0.1333	0.1	0.0333
0.1	1.0	0.576	0.1	0.476
0.1	10.0	5.075	0.1	4.975
[P] = 0.1	0	0	[P] = 0.1	0

Solving for x, we obtain

$$x = \frac{b^2}{c + 2b} \qquad ...(1)$$

Equation (1) says that the amount of NaCl, x, diffused from right to left is inversely proportional to the concentration of the non-diffusible ion (P^-), c, in the left compartment. This unequal distribution of the diffusible ions (Na^+ and Cl^-) in the two compartments is the result of the Donnan effect.

The osmotic pressure of the protein solution is now determined by the *difference* between the number of particles in the left compartment and that in the right compartment. We write

$$\pi_3 = \underset{\text{left compartment}}{\{(c + c + x + x)} - \underset{\text{right compartment}}{2(b - x)\}} \, RT = (2c + 4x - 2b) \, RT$$

From Eq. (1), We have

$$\pi_3 = 2c + \left(\frac{4b^2}{c + 2b} - 2b\right) RT = \left(\frac{2c^2 + 2cb}{c + 2b}\right) RT$$

Two limiting cases may be applied to the equation above. If $b << c$, $\pi_3 = 2cRT$, which gives the same result as Case 2. On the other hand, if $b >> c$, $\pi_3 = cRT$, which is identical to Case 1. The important conclusion we arrive at is that the presence of NaCl in the right compartment decreases the osmotic pressure of the protein solution compared to Case 2 and therefore minimizes the Donnan effect. When a very large amount of NaCl is present, the Donnan effect can be completely eliminated. In general, we have $\pi_1 \leq \pi_3 \leq \pi_2$. Since proteins are usually studied in buffer solutions that contain ionic species, the osmotic pressure measured will be less than the case of using pure water as the solvent. Table 3.7 shows the Donnan effect for the NaCl example at several concentrations and the corresponding osmotic pressure at 298 K.

An alternative approach to eliminate the Donnan effect is to choose a pH at which the protein has no net charge, called the *isoelectric point*. At this pH, the distribution of any diffusible ion will always be equal in both compartments. This method is difficult in practice because most proteins are least soluble at their isoelectric points.

We have simplified the discussion of the Donnan effect by assuming no change in either pH or volume of the solution. Also, for the sake of simplicity, we have used a common diffusible ion,

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Na^+, in deriving Eq. (1). A Donnan effect does not depend on simple conditions. It would still be observed if we started with K^+P^-, and NaCl, although the treatment would become more complicated.

The Donnan effect is essential to understand the distribution of ions across the membranes of living organisms and membrane potentials. A particularly important case is the distribution of bicarbonate and chloride ions between plasma water and red-blood-cell water.

3.11 Equilibrium Dialysis

Dialysis is the process of removing small ions and other solute molecules from a protein solution. Suppose that in an experiment we have precipitated hemoglobin from a solution by the salting-out technique using ammonium sulfate. The protein can be free of the $(NH_4)_2SO_4$ salt as follows. First, the precipitate is dissolved in water or, more frequently, in a buffer solution. The protein solution is then placed in a cellophane bag, which in turn, is immersed in a beaker containing the same buffer (Figure 3.7). Since both the NH_4^+ and SO_4^{2-} ions are small enough to diffuse through the membrane but not the protein molecules, the ions in the bag will begin to enter into the outside solution as a result of the unequal chemical potentials :

$$(\mu NH_4^+)_{inside} > (\mu NH_4^+)_{outside}$$
$$(\mu SO_4^{2-})_{inside} > (\mu SO_4^{2-})_{outside}$$

This process continues until the chemical potentials of each type of ions become equal inside the outside of the bag and an equilibrium is established. It is possible to remove all the $(NH_4)_2SO_4$ by continually changing the buffer solution in the beaker.

The procedure described above may be reversed to study the binding of ions or small ligands to proteins. In this case we begin by placing the *pure* protein solution in the cellophane bag (called phase 1), which is then immersed in a buffer solution (called phase 2) that contains the ligands (L) of known concentration. At equilibrium, the chemical potentials of the free (unbound) ligands in both phases must be the same (Figure 3.8) so that

$$(\mu_1)_1^{unbound} = (\mu_1)_2^{unbound}$$

or

$$(\mu^\phi + RT \ln a_L)_1^{unbound} = (\mu^\phi + RT \ln a_L)_2^{unbound}$$

Since the standard chemical potential μ^ϕ is the same, we have

$$(a_L)_1^{unbound} = (a_L)_2^{unbound}$$

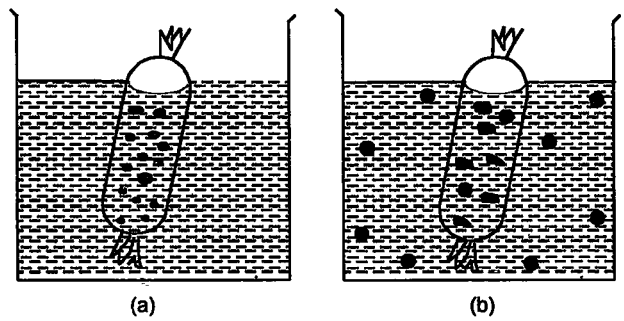

Fig. 3.7. Arrangement for dialysis. The small dots denote ions and the large dots proteins (*a*) At the start of dialysis. (*b*) At equilibrium, most of the small ions have diffused out of the cellophane bag. By repeatedly replacing the buffer solution in the beaker, it is possible to remove all of the small ions not bound to the protein.

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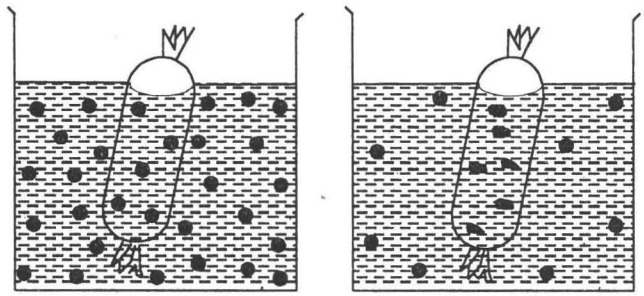

Fig. 3.8. Arrangement for studying equilibrium dialysis. The small dots denote ligands and the large dots proteins. (*a*) Initially, a cellophane bag containing a protein solution is immersed in a buffer solution containing the ligand molecules. (*b*) At equilibrium, some of the ligand molecules have diffused into the bag and complexed with the protein molecules.

If the solutions are dilute, we can replace activities with concentrations so that

$$[L]_1^{\text{unbound}} = [L]_2^{\text{unbound}}$$

However, the total concentration of the ligands inside the bag is given by

$$[L]_1^{\text{total}} = [L]_1^{\text{bound}} + [L]_1^{\text{unbound}}$$

Therefore, the concentration of ligands bound to protein molecules is

$$[L]_1^{\text{bound}} = [L]_1^{\text{total}} - [L]_1^{\text{unbound}}$$

The first quantity on the right-hand side of the last equation can be determined by analyzing the solution in the bag *after* its removal from the beaker; the second quantity is obtained by measuring the concentration of the ligands in the solution remaining in the beaker. (Remember that the concentrations of the unbound ligands are equal in phases 1 and 2). We can now see how both the intrinsic dissociation constant K and the number of binding sites n can be obtained by using the equilibrium dialysis technique. Suppose that we start with a protein solution of known concentration in phase 1 and ligand solution of known concentration in phase 2. At equilibrium, the quantity γ is given by

$$\gamma = \frac{[L]_1^{\text{bound}}}{[P]^{\text{total}}}$$

where $[P]^{\text{total}}$ is the concentration of the original protein solution. The experiment can be repeated by using different concentrations for the protein and ligand solutions and the values of K and n can be determined from either the Hughes Klotz plot or the Scatchard plot.

The equilibrium dialysis technique has been successfully employed in the binding of drugs, hormones, and other small molecules to proteins and nucleic acids. It is useful to keep in mind that our discussion implicitly assumes that the ligand is a non-electrolyte so that the concentrations of unbound ligands are equal in phases 1 and 2 at equilibrium. If the ligand is an electrolyte, we must apply the Donnan equilibrium condition for treating the dialysis data.

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4 Bioenergetics

4.1 Introduction

The study of energy changes accompanying biochemical reactions is termed as bioenergetics or biochemical thermodynamics. These reactions are accompanied by the liberation of energy as the reacting system moves from a higher to a lower energy level. Generally the energy liberated is in the form of heat. But most of biochemical reactions are isothermic. Therefore, it is not possible to use the heat liberated to drive the vital processes that require energy. These processes (*e.g.*, synthetic reactions, muscular contraction, nerve conduction, and active transport) obtain energy by chemical or coupling to oxidative reactions.

In the living systems, the energy is stored in the form of energy rich or high energy compounds which are mainly phosphates containing molecules. Whenever the energy is needed by metabolic process, these high energy compounds which are mainly phosphates containing molecules. Whenever the energy is needed by metabolic process, these high energy compounds undergo decomposition and the energy liberated is utilised. Further, the syntheses of these compounds take place as result of various metabolic activities. For example, the oxidation of NADH in the mitochondrion is the main reaction.

4.2 Importance of Bioenergetics

Suitable fuel is required to provide the energy that enables the animal to carry out its normal process. How the organism obtains this energy from its food is basic to the understanding of

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normal nutrition and *metabolism*. Death from *starvation* occurs when available energy reserves are depleted, and certain forms of malnutrition are associated with energy imbalance (*marasmus*). The rate of energy release, measured by the metabolic rate, is controlled by the *thyroid hormones, whose malfunction is a cause of disease*. Storage of surplus energy results in *obesity*, one of the most common diseases of occidental society.

4.3 Energy and its Various Forms

Energy of a system may be defined as the capacity of a system to do work. This capacity may be bound in the molecules. The bound form of energy is termed as *potential energy*. Potential energy is generally measured in terms of work that could be undertaken when the energy were released. Potential energy may be of various types such as electrical, chemical, photic (light), atomic or positional. In the biological world, the main source of potential energy is the solar energy which gets fixed up in the form of chemical energy of carbohydrate molecules during photosynthesis in green plants. Other organisms have to depend directly or indirectly upon this process (photo synthesis) for a constant supply of energy to maintain their structures and to perform their numerous functions. Energy is not only required for mechanical work, maintenance of body temperature and osmotic work but also to drive numerous synthetic reactions.

The molecules in motion possess capacity to carry out work. The energy possessed by a molecule or a body by virtue of its motion is termed as *kinetic energy*. This type of energy depends upon the mass of body and its velocity of motion in accordance to the following relation :

$$\text{K.E.} = \frac{1}{2}mv^2$$

where K.E. represents the kinetic energy, m the mass of the body and v the velocity of the body.

Other forms of energy are as follows :

Free energy. See Art. 4.3

Internal energy. See Art 4.3

The Joule is the unit of energy and is numerically equivalent to 0.239 calorie. One calorie is equal to 4.184 Joules (J) and one Kilocalorie is equal to 4.184 Kilojoules (KJ).

4.4 Principles of Thermodynamics

Biological systems conform to the general laws of thermodynamics.

Many quantitative observations carried out on the interconversion of various forms of energy have helped scientists to the formulation of two fundamental laws of thermodynamics, the first and second. These laws have been found to be useful in understanding :

(*a*) the direction of a reaction, whether from left to right or vice versa,

(*b*) the accomplishment of work, whether useful or not, and

(*c*) whether the energy for driving a reaction must be delivered from an external source.

1. First law of thermodynamics : The principle of conservation of energy which is based on cumulative experience of ages can be stated as :

"Energy may be converted from one form to another, but it is impossible to create or destroy it."

Previously, different attempts were made to disprove this principle by constructing a machine that may continue to operate without any expenditure of fuel and may thus create energy out of nothing (*Perpetual motion machine of first kind*). But all attempts failed. Therefore, the net result is that the principle of conservation of energy is universally accepted and is retitled as the *first law of thermodynamics*.

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There are various ways of enunciating the first law of thermodynamics. Here some of these are given as follows :

(*i*) *First statement :* It may be put as :

"*Energy of an isolated system must remain constant, although it may be transformed from one form to another.*"

As heat is given out to the surroundings in exothermic reactions, the internal energy of reacting system decreases while the energy of surroundings increases correspondingly. Therefore, the total energy of the system and the surroundings (*i.e.*, isolated system) taken together remains unchanged.

As heat is absorbed from the surroundings in exothermic reactions, the internal energy of the reacting system increases while the energy of the surroundings decreases correspondingly. Therefore, the total energy of the system and the surroundings taken together remains unchanged.

(*ii*) *Second statement :* It is as below :

"*Energy in one form, if it disappears, will make its appearance in an exactly equivalent quantity in another form.*"

For example, coal contains inherently a lot of internal energy. During burning this energy functions as chemical energy and yields heat. The heat is converted into the mechanical energy of the steam, which makes the engine to move a train. In this example, energy is disappearing in one form and appearing in another form in an exactly equivalent quantity.

Mathematical formulation of the first law : Consider a system whose state on absorbing a quantity of heat '*q*' changes from A to B (Fig. 4.1). This heat is used in two ways :

(*i*) In increasing the internal energy of the system, *i.e.*,

$$\Delta E = E_B - E_A$$

where E_A and E_B denote the internal energies associated with the system in its states A and B respectively, and

(*ii*) To do some external work '*w*' by the system on its surroundings.

From the first law, we get

Heat absorbed by the system = Increase in its internal energy + Work done by the system

$$\therefore \quad q = \Delta E + w$$

$$\Delta E = q - w \qquad ...(1)$$

Differential form of the first law : For an infinitesimal process, Eq. (1) may become as follows :

$$dE = \delta q + \delta w \qquad ...(2)$$

where an infinitesimal process may be defined as a process involving only infinitesimal changes in thermodynamic variable defining the system. In Eq. (2) the differential is distinguished in notations by *d* from δ. This can be understood by the following discussion.

(A) Change in internal energy is independent of the path taken : The change in internal energy is dependent

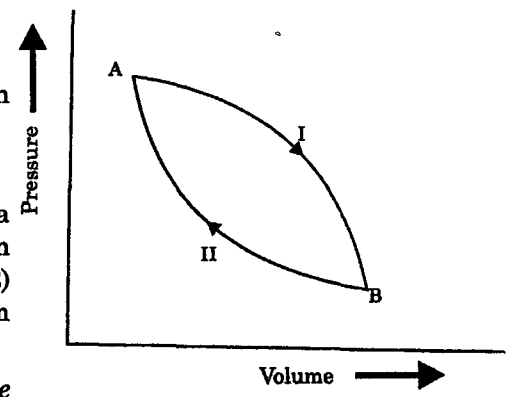

Fig. 4.1. Change of internal energy.

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on path by which it is accomplished and the previous history of the system. Thus, change in internal energy is an exact differential. So in mathematical terms, an exact differential is always denoted by a notation *d, e.g., d*E in equation (2).

(B) Nature of δq *and* δw : The '*q*' and '*w*' are not state functions because changes in their magnitude for a process depend upon :

(*i*) the path by which the change is accomplished, and

(*ii*) the previous history of the system.

This fact can be understood from Fig. 4.2. The work done in passing from state A to state B by four different paths has been illustrated in Fig. 4.2. The value of *w* will be equal to the area under the curve in a P – V diagram which is shown by shaded regions in Fig. 4.2 for the various paths. It is seen that the work done in passing from A to B by different paths is not same. It means that *w* is not a definite quantity.

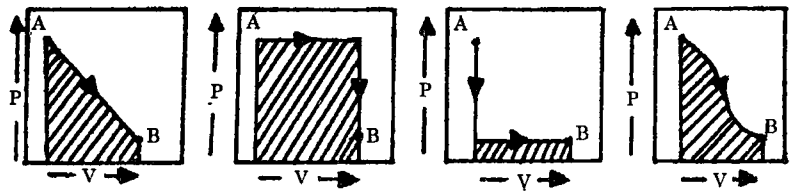

Fig. 4.2. Dependence of the work done on the path followed.

From the above discussion, it follows that *w* and *q* are not definite quantities. Mathematically, infinitesimal changes in *q* and *w* are not exact differentials but are inexact differentials.

In mathematical language, we always write the inexact differential by δq, δw, etc., [Eq. (2)].

Sign of q : When heat is absorbed or gained by the system, *q* is + ve. When heat is evolved or lost by the system, *q* is –ve.

Sign of w : When work is done by the system, the value of *w* is +ve. When work is done on the system, the value of *w* is –ve.

Sing of ΔE : When E_2 (the energy in the final state of the system) is greater than E_1 (the energy in the initial state of the system), the increase in energy (ΔE) is positive but when E_2 is less than E_1, then ΔE is negative and represents decrease in energy.

Significance of the first law of thermodynamics : This law establishes an exact relation between heat and work. It tells us that a certain quantity of heat will produce a definite amount of work and *vice-versa*. The first law of thermodynamics denies that work or energy can be produced from nothing. Work cannot appear without disappearing of heat.

Modification : The discovery of Albert Einstein that mass is convertible into energy, however, makes it essential to make a change in the definition of the first law of thermodynamics. It is possible to convert mass into energy in accordance to the following equation :

$$E = mc^2$$

where *E* is the energy produced by the destruction of mass *m* and *c* is the velocity of light. The modified first law of thermodynamics may be stated as follows :

"The total mass and energy of an isolated system remains unchanged."

Final remark : As matter to energy or energy to matter conversions are of little importance in the biological systems, it means that the first law of thermodynamics has not valid applications in biochemistry.

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Limitations of first law of thermodynamics : There are three main limitations of first law of thermodynamics. These are as follows :

(*a*) The first law of thermodynamics simply establishes equivalence between different forms of energy. *However, this law does not tell us under what conditions and to what extent it is possible to bring about conversion of one form of energy into the other.* This can be understood from the following examples :

(*i*) We know that heat is flowing from a hot to cold body. First law simply tells us that the quantity of heat lost by the hot body is equal to the heat gained by the cold body. It never tells us that heat can only flow from the hot to the cold body and not in the reverse direction.

(*ii*) Zinc dissolves spontaneously in sulphuric acid, a definite amount of energy being liberated. The reverse process is not spontaneous. Without external influence, the reaction proceeds in only one direction.

From the above examples we can predict that there must be some laws besides the first law which should form the basis of predicting the direction of a certain reaction to occur, and, if so, and to what extent. This, however, comes within the scope of the second law of thermodynamics.

(*b*) The first law of thermdynamics is a qualitative statement which does not prelude the existence of either a heat engine or a refrigerator. The first law does not contradict the existence of a 100% efficient heat engine or a self-acting refrigerator. In practice, these two are not attainable. These phenomena are recognised and this led to the formulation of a law governing these devices. It is called the second law of thermodynamics.

(*c*) The first law fails to explain why chemical reactions do not proceed to completion. For example, consider the reaction $CO\ (g) + H_2O\ (g) \rightleftharpoons CO_2\ (g) + H_2(g)$. If originally one mole of CO and one mole of H_2O were in the reaction vessel, then if the reaction went to completion, one mole of CO_2 and one mole of H_2 would be produced. If the reaction vessel is examined at equilibrium, the yield is less than 100%. However, this comes within the scope of second law of thermodynamics.

Internal energy : It is the energy associated with a system by virtue of its molecular constitution and the motion of its molecules. The contribution of energy due to molecular constitution is known as *internal potential energy* and the contribution of energy due to the motion of molecules is known as *internal kinetic energy*. Hence the total internal energy of a system is given by the sum of two types of energies, *i.e.*,

$$\text{Total internal energy} = \text{Internal potential energy} + \text{Internal kinetic energy}$$

A fixed quantity of any substance, under a given set of conditions, has got a definite amount of internal energy associated with it. This amount is different for different substance. It is, therefore, regarded as an *extensive property.*

Internal energy is symbolized by E *or* U.

2. Second law of thermodynamics : All the neutral processes tend to have a tendency to go to equilibrium. The second law of thermodynamics is related to the equilibrium state of a process.

Second law of thermodynamics is a generalisation of certain experiences about heat engines and refrigerators. It has been stated in a number of ways, but all the statements are logically equivalent to one another.

(*a*) *Kelvin-planck statement : "It is impossible to obtain a continuous supply of work from a body or engine that can transfer heat from a single heat reservoir."*

The above statement is a negative statement. By this statement, it means that a single reservoir at a single temperature cannot continuously transfer heat into work. It means that

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there should be two reservoirs for any heat engine. One reservoir (called the *source*) is taken at a higher temperature while the other reservoir (*called the sink*) is taken at a lower temperature.

According to this statement, zero degree absolute temperature is not attainable as no heat is rejected to the sink at zero degree Kelvin. Suppose an engine works between any temperature higher than zero degree Kelvin. It implies that it uses a single reservoir which contradicts the Kelvin-Planck's statement of the second law. Similarly, it can be said that no engine can be 100% efficient.

(*b*) *Clausius statement* : Heat flows from hot to cold body due to difference of temperature. However, in a refrigerator, heat gets absorbed from a body at lower temperature and gets absorbed from a body at lower temperature and gets rejected to a body at higher temperature. Here heat is being flown through some outer agency, *i.e.*, it is not feasible to cause heat to flow from a colder to a hotter body without absorbing from some external source. This led Clausius to state the second law of the thermodynamic in the following words : "*It is impossible to transfer for a self-acting machine, unaided by any external agency to convert heat continuously, from one body at lower temperature to a body at a higher temperature.*"

(*c*) *Planck statement* : "*It is impossible to construct a device that will work in a complete cycle and convert heat into work without making any change in the surroundings.*"

(*d*) *Thomson statement* : It a heat engine, the working substance taken heat from hot body; a part of it is converted into work while remaining is given to the sink. No engine has so far been constructed which continuously takes heat from a single body and changes the whole of it to work without making any change in the system. Hence the presence of a cold body is a necessity for converting heat into work. This led Thomson to state that "*it is impossible to obtain a continuous supply of work by cooling a body to a temperature lower than that of coldest of its surroundings.*"

(*e*) *Diffusion of a gas always takes place from a region of higher to one at a lower pressure, until the pressure becomes uniform.*

Thus, the second law of thermodynamic may be stated in various ways. All the statements, appear to be different from one another. But a closer look at them, reveals that they are all the modified forms of the same fundamental idea that, "*all forms of energy are convertible into heat but the heat so obtained cannot be converted into other forms of energy by any process.*"

The difference between the first and second laws of thermodynamics lies in the fact that the *first law* is concerned with the accounting of the various kinds of energy involved in a given process, while the *second law* is concerned with the availability of the energy of a given system for doing useful work.

4.5 Entropy

The second law of thermodynamics introduces the concept of *entropy* which may be regarded as the amount of energy unavailable for conversion into work.

The term entropy was introduced by Clausius (1865) and is denoted by symbol S. In simple words, entropy of a system may be regarded as the amount of energy unavailable for conversion into work. It is, however, very difficult to define actual entropy of a system precisely. Therefore, more generally the change in the entropy of a system is defined rather than the absolute entropy of the system.

The increase in entropy dS in the course of an infinitesimal change is equal to δq_{rev} the heat absorbed when the change is carried out in a reversible manner, divided by the absolute temperature T, *i.e.*,

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$$dS = \frac{\delta Q_{rev}}{T} \quad ...(1)$$

Although δQ_{rev} is not an exact differential, it has a definite value for a reversible, isothermal change, and dS as defined by equation (1) is an exact differential. The entropy S of a system is thus a state function.

If heat change takes place at different temperatures, then we have

$$dS = \frac{\delta Q_1}{T_1} + \frac{\delta Q_2}{T_2} + \frac{\delta Q_3}{T_3} + ... = S\frac{\delta Q_{rev}}{T} \quad ...(2)$$

Eq. (1) may be written as follows:

$$\int dS = \int \frac{\delta Q_{rev}}{T}$$

or

$$S = \int \frac{\delta Q_{rev}}{T} \quad ...(3)$$

From equation (3) it means that "*the entropy difference between two states of a given system is equal to the summation of all the* Q_{rev}/T *terms of all the steps which are necessary to bring about a reversible transformation between these two states, where* q_{rev} *denotes the heat absorbed reversibly and T the corresponding temperature.*"

Units of entropy : For a finite change at constant temperature dS *becomes* ΔS, δQ_{rev} becomes q_{rev} and Eq. (1) becomes as follows :

$$\Delta S = \frac{Q_{rev}}{T}$$

$$\Delta S = \frac{\text{Heat change}}{\text{Absolute temperature}}$$

$$= \frac{J}{K} = JK^{-1}$$

ΔS is an extensive property and its value, therefore, depends upon the amount of substance involved. Therefore, the unit of entropy will be $JK^{-1}\ mol^{-1}$ (S.I. units).

In both physical and biological world, the entropy is always changing. Whenever there occurs spontaneous change in a process the entropy increases.

Physical significance : (*i*) A characteristic of a spontaneous process (such as the flow of heat in a metal base from the hot to the cold end, diffusion of one gas into another, the dissolution of a solid by a solvent etc.), is that it is accompanied by an increase in the 'disorder' or 'randomness' of the molecules constituting the system. In all such processes, moreover there occurs an increase of entropy. Hence entropy may be regarded as "*a measure of disorder or randomness of the molecular arrangements in a system.*"

(*ii*) A spontaneous process always proceeds from a less probable state to a more probable state by the interplay of natural forces. We know that a spontaneous process is always accompanied by an increase in the entropy, we can therefore identify the entropy as a function of the probability of the thermodynamic state. Since we know that both the entropy, and the thermodynamic probability increase simultaneously in a process we can therefore consider the state of equilibrium as the state of maximum probability. It shows that a system, when left to itself, tends to attain a state of equilibrium.

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Entropy in Biological Systems

Living organisms are able to maintain their organisation by taking in free energy of nutrients (or sunlight) from the environment and they return an equal amount of energy in a less useful or useless form to their environment. This energy is mainly in the form of heat that gets randomised throughout the rest of the universe. In other words, it implies that living organisms constantly produce entropy by dissipating useless energy in their surroundings to maintain their internal organisation.

This can be understood from the following two examples :

1. Oxidation of glucose in respiration : The living organisations oxidise glucose with atmospheric oxygen to produce carbon dioxide, water and energy.

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O + \text{Energy} \begin{cases} \nearrow \text{Heat} \\ \searrow \text{ATP} \end{cases}$$

The energy is conserved in the form of ATP which is used in various endergonic biochemical reactions. However, heat produced is less useful energy and as a result the entropy increases. In this process there occurs a change in the state of matter. As a result the entropy also increases. Furthermore there also occurs an increase in number of molecules. For example, one solid glucose molecule, on oxidation with 6 gaseous oxygen molecules yields 6 liquid water and 6 gaseous carbon dioxide molecules. Thus, from a total of 7 reactant molecules (1 + 6), 12 molecules (6 + 6) of products are formed. Whenever then occurs an increase in the number of molecules in a process, molecular disorder or entropy increases.

It is possible to calculate the actual amount of energy contributing towards the entropy during the oxidation of glucose by using the following bioenergetic equation :

$$\Delta G = \Delta H - T\Delta S$$

where ΔS can be calculated from this equation if the values of ΔG, ΔH and T are known. At constant temperature (298 K) and pressure (1 atm), the various values of oxidation of glucose are as follows :

$$\Delta G = -686{,}000 \text{ cal/mole}$$

$$\Delta H = -673{,}000 \text{ cal/mole}$$

$$T = 298 \text{ K}$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} = \frac{-673000 - (-686{,}000)}{298 \text{ K}}$$

$$= \frac{13000}{298} = 43.62 \text{ cal/mole.}$$

Thus, during the oxidation of one mole of glucose the entropy of the universe increases by 43.62 cal/mole.

2. Entropy effects in enzyme-catalysed reactions : Enzymes are known to catalyse the conversion of the substrate to the product after binding with the substrate molecule. When a substrate molecule undergoes binding with the active site of the enzyme, most of the internal entropy of the substrate is lost. Because of binding, the substrate molecule is not free to rotate or move. This binding and decrease in entropy contribute towards an increase in "effective concentration" of the substrate molecule. This entropy effect is partly responsible for the rate enhancement effect of the enzymes. Also, from a bimolecular state a unimolecular state is achieved during the formation of enzyme-substrate complex.

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$$E + S \longrightarrow \underset{\text{Complex}}{ES}$$

This also brings about a decrease in entropy because the number of free molecules decreases.

In physical as well as biological world, entropy is always changing. Whenever there occurs a spontaneous change in a process, there occurs entropy change and there also occurs an increase in disorder of system.

4.6 Free Energy

In biological systems, the free energy is the most important type of energy.

It is denoted by G. It may be regarded as a measure of the potential energy of the substance but it is a quantity that cannot be measured directly. However, its change accompanying chemical reaction or a process can be measured. It is denoted by ΔG. *The change in free energy* (ΔG *is that portion of the total energy change in a system which is available for doing work, i.e., it is the useful work*). The change in free energy ΔG of a reaction is calculated by taking the difference between the sum of the free energies of the products and that of reactants.

$$A + B \rightleftharpoons C + D \qquad ...(1)$$

$$\Delta G = \text{Free energy} = (G_C + G_D) - (G_A + G_B) \qquad ...(2)$$

When the free energy change in a reaction is negative (ΔG negative), the reaction is called *exergonic* and when the free energy change is positive (ΔG positive), the reaction is called *endergonic.* An example of exergonic process is the process of respiration while an example of endergonic process is the process of photosynthesis.

It is often said that the reactions with a negative ΔG can occur spontaneously but the sign and magnitude of ΔG give no information about the rate of a reaction. Thus, for the complete oxidation of a glucose molecule according to the equation,

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O$$

$\Delta G = -2870$ kJ per mole of glucose; in spite of this large negative ΔG, glucose is quite stable in air and the rate of spontaneous reaction being negligibly small. Further if ΔG is zero, a state of chemical equilibrium exists and no net chemical change takes place.

4.7 Relation between ΔG and ΔS

OR

Combination of First and Second law of Thermodynamics

As the entropy changes of chemical reactions could not be readily measurable, the entropy is not regarded as criterion whether a biochemical process can take place spontaneously or not. Furthermore, for spontaneity, both the entropy changes (that of the surroundings and of the system) should be known. These difficulties could be overcome by using a different thermodynamic function, called the *free energy,* which is denoted by the symbol G. (In older literature, however, the symbol F is used in its place).

The symbol G has been given in honour of Josiah Willard Gibbs (1839–1903), an American physical chemist, who single-handedly created a large portion of chemical thermodynamics.

In 1878, Gibbs introduced the free energy function by combining the first and second laws of thermodynamics in the form of following equation :

$$\Delta G = \Delta H - T\Delta S \qquad ...(1)$$

where, ΔG = the change in free energy of a creative system,

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ΔH = the change in heat content or enthalpy of this system,

T = the absolute temperature at which the process is taking place, and

ΔH = the change in entropy of this system.

In fact, this equation gives quantitative interrelationship between the changes in energy, heat and entropy in chemical reactions taking place at constant temperature (T) and pressure (P), the conditions prevailing in biological systems.

The term $T\Delta S$ is that fraction of ΔH which cannot be converted into useful work. The ΔG reveals the free energy change or the theoretically available useful work. Naturally, in most cases the system has been 'inefficient' and not all of the theoretically available work can be utilised. However, the properties of the surroundings do not take part into this process :

The enthalpy change, ΔH is given by the following equation :

$$\Delta H = \Delta E + p\Delta V \qquad ...(2)$$

where, ΔE = the change in internal energy of a reaction, and

ΔV = the change in volume of this reaction.

As the volume changes, ΔV has been found to be small for nearly all biochemical reactions, hence ΔH has been nearly equal to the change in internal energy, ΔE. Therefore, Equation (3) becomes as follows :

$$\Delta G \approx \Delta E - T\Delta S \qquad ...(3)$$

Thus, the change in free energy of a reaction, ΔG will depend both on the change in internal energy and on the change in entropy of the system. The ΔG has been a valuable criterion in determining whether a reaction can take place spontaneously or not. Thus,

(*i*) If ΔG if negative, the reaction takes place spontaneously with loss of free energy, *i.e.*, it is *exergonic*. If, in addition ΔG is of great magnitude, the reaction goes virtually to completion and has been essentially irreversible.

(*ii*) If, however, ΔG is positive, the reaction proceeds only if free energy can be gained, *i.e.*, it is *endergonic*. If, in addition ΔG is of high magnitude, the system is stable having little or no tendency for a reaction to take place.

(*iii*) If ΔG is zero, the system is at equilibrium and no net change takes place. It means that the system is stable with little or no tendency for a reaction to occur.

With regard to the free energy change, ΔG of a reacting system two more points have to be understood.

I. Firstly, the ΔG of a reaction is dependent only on the free energy of the products minus that of the reactants. *The ΔG of a reaction is independent of the path of transformation.* Obviously, it means that the mechanism of a reaction has no effect on ΔG. As an instance, the value of ΔG has been the same for the oxidation of glucose to CO_2 and H_2O whether it occurs by combustion or by a series of enzyme-catalysed reactions.

II. Secondly, *the value of ΔG gives no information about the rate of a reaction.* A negative ΔG reveals that a reaction can take place spontaneously, but it does not signify that it will take place at perceptible rate. As we already know that, the rate of reaction rather depends on the free energy of activation ($\Delta G = \neq$), which is unrelated to ΔG.

In order to get a feeling for the magnitude of changes in various forms of energy, an actual example of aerobic oxidation of glucose may be taken into consideration. The living cells carry out oxidation of glucose in the presence of oxygen to CO_2 and H_2O at constant temperature and pressure.

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$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O$$

If we assume that the temperature is 25°C (or 298 K) and the pressure is 1.0 atm. (or 760 mm Hg), which are standard conditions in thermodynamic calculations, the following energy changes occur per molecule of glucose oxidised—

$\Delta G = -686{,}000$ cal/mol (*i.e.*, the free energy of the reacting molecules has decreased)

$\Delta H = -673{,}000$ cal/mol (*i.e.*, the reacting molecules have released heat)

Eq. (1) may also be written as :

$$\Delta S = \frac{\Delta H - \Delta G}{T} \qquad ...(4)$$

On substituting the above values in Eq. (4), we get

$$\Delta S = \frac{-673{,}000 - (-686{,}000)}{298}$$

$= 44$ cal/deg (*i.e.*, the entropy of the universe has increased)

$= 184.096$ j/deg.

4.8 Relationship between Standard Free Energy Change and Equilibrium Constant

In a model reaction

$$A + B \rightleftharpoons C + D \qquad ...(1)$$

the free energy change, ΔG of this reaction will be given by

$$\Delta G = \Delta G^0 + RT \log_e \frac{[C][D]}{[A][B]} \qquad ...(2)$$

where, ΔG^0 = Standard free energy change

R = Gas constant

T = Absolute temperature

[A], [B], [C] and [D] = Molar concentrations (*i.e.*, activities) of the reactants.

The standard free energy change (ΔG^0) of a chemical reaction may be defined as the free energy change when the reactants and products are in their standard states (298 K, 1 atm pressure).

Thus, the ΔG of a reaction depends on the nature of the reactants (expressed in ΔG^0 term) and on their concentrations (expressed in logarithmic terms), as shown in Eq. (2).

At equilibrium, $\Delta G = 0$. Equation then becomes as follows :

$$0 = \Delta G^0 + RT \log_e \frac{[C][D]}{[A][B]} \qquad ...(3)$$

or

$$\Delta G^0 = -RT \log_e \frac{[C][D]}{[A][B]} \qquad ...(4)$$

The equilibrium constant under standard conditions, K'_{eq} for the reaction $A + B \rightleftharpoons C + D$, will be given as follows :

$$K'_{eq} = \frac{[C]_{eq}\,[D]_{eq}}{[A][B]}$$

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At equilibrium, there is no change in free energy ($\Delta G = 0$); therefore, Eq. (4) becomes as follows :

$$0 = \Delta G^0 + RT \ln \frac{[C]_{eq}\,[D]_{eq}}{[A]_{eq}\,[B]_{eq}}$$

$$0 = \Delta G^0 + RT \ln K'_{eq} \text{ [Use Eq. (4)]}.$$

By changing logarithms to the base 10, the above equation becomes as follows :

$$0 = \Delta G^0 + 2.303\ RT \log K'_{eq} \quad ...(5)$$

$$\Delta G^0 = -2.303\ RT \log K'_{eq} \quad ...(6)$$

Eq. (6) reveals that if it is possible to determine equilibrium constant for any reaction, the standard free energy change can also be calculated. Conversely if ΔG^0 is known, the actual free energy ΔG for any reaction can also be calculated.

Eq. (6) can be put in the following form :

or $$K'_{eq} = 10^{-\Delta G^0/(2.303RT)} \quad ...(7)$$

Substituting $R = 1.98 \times 10^{-3}$ kcal mol^{-1} $degree^{-1}$ and $T = 298^0\ K$ (corresponding to 25°C) gives

$$K'_{eq} = 10^{-\Delta G^0/1.36} \quad ...(8)$$

when ΔG^0 has been expressed in kcal/mol. Hence the standard free energy, ΔG^0 and the equilibrium constant, K'_{eq} are related by a simple expression. For example, a change in equilibrium constant by a factor of 10 causes a change in standard free energy of 5.69 kj/mol at 25°C (refer Table 4.1). At 37°C, however, the change in standard free energy would be of –5.69 kj/mol. Values of ΔG^0 may be expressed in joules or calories per mole.

Table 4.1

NUMERICAL RELATIONSHIP BETWEEN EQUILIBRIUM CONSTANTS OF CHEMICAL REACTIONS AND THEIR STANDARD FREE ENERGY CHANGES AT 25°C

K'_{eq}	ΔG^0 (*kcal/mol*)	ΔG^0 (*kJ/mol*)
10^{-5} or 0.00001	+ 6.82	+ 28.53
10^{-4} or 0.0001	+ 5.46	+ 22.84
10^{-3} or 0.001	+ 4.09	+ 17.12
10^{-2} or 0.01	+ 2.73	+ 11.42
10^{-1} or 0.1	+ 1.36	+ 5.69
1	1	0
10^{1} or 10	– 1.36	– 5.69
10^{2} or 100	– 2.73	– 11.42
10^{3} or 1,000	– 4.09	– 17.12
10^{4} or 10,000	– 5.46	– 22.84
10^{5} or 1,00,000	– 6.82	– 28.53

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If ΔG^0 is negative (*i.e.*, the products have less free energy than the reactants), the reaction will take place to form the products under standard conditions, as all chemical reactions will tend to go in that direction causing a decrease in the free energy of the system. If ΔG^0 is positive (*i.e.*, the products of the reaction contain more free energy than the reactants), the reaction will proceed in the reverse direction if we start with 1M concentration of all components. To be more explicit, reactions having a negative ΔG^0 take place forward in the direction written when they start with all reactants and products at 1.0 M, until they reach equilibrium. Reactions with a positive ΔG^0 will take place in the reverse of the direction written when they start with all components at 1.0 M. Table 4.2 summarises these relationships.

Table 4.2

RELATIONSHIP AMONG K'_{eq}, ΔG^0 AND THE DIRECTION OF CHEMICAL REACTIONS UNDER STANDARD CONDITIONS

When K'_{eq} is	*ΔG is*	*Starting with 1 M components, the reaction*
> 1.0	Negative	Proceeds forward
1.0	Zero	Remains at equilibrium
< 1.0	Positive	Proceeds backward

Two more points should be understood :

I. Biochemical reactions occur near pH 7.0. Hence, pH 7.0 is conventionally designated the standard pH in biochemical energetics. The standard free energy change at pH 7.0 in biochemical energetics has been designated by the symbol, ΔG^0.

II. The basic SI (Syste'me International) unit of energy is joule (J). However, in biology and medicine, heat and energy measurements are equally expressed in calories (cal). The interrelationship between calories and joules is :

$$1.000 \text{ cal} = 4.184 \text{ J}.$$

4.9 Biochemist's Standard State

It is important to first distinguish between the *standard states* employed by physical chemists and biochemists. In physical chemistry, the standard state refers to the situation in which all the reactants and products are at unit molar (or molal) concentration. In biochemistry, we follow the same procedure except that we define the hydrogen-ion concentration for the standard state to be 10^{-7} M because the physiological pH is about 7. Consequently, the change in the standard Gibbs free energy according to these two conventions will be different for reactions involving uptake or liberation of hydrogen ions. We shall therefore replace ΔG^0 with $\Delta G^{0'}$ in discussing biochemical process. Consider the reaction

$$A + B \longrightarrow C + xH^+$$

The Gibbs free energy change (ΔG) for the process is given by

$$\Delta G = \Delta G^0 + RT \ln \frac{\dfrac{[C]}{1\text{ M}} \dfrac{[H^+]}{1\text{ M}}}{\dfrac{[A]}{1\text{ M}} \dfrac{[B]}{1\text{ M}}}$$

where 1 M represents the physical chemist's standard state of solute in solution. Since the biochemist's standards state for H^+ ions is 10^{-7} M, the Gibbs free energy change for the same process is now given by

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$$\Delta G = \Delta G^0 + RT \ln \frac{\frac{[C]}{1\ M} \frac{[H^+]}{10^{-7}\ M}}{\frac{[A]}{1\ M} \frac{[B]}{1\ M}}$$

Note that regardless of the convention for the standard state, ΔG must remain unchanged. From the last two equations we obtain

$$\Delta G^0 = \Delta G^{0'} + xRT \ln \frac{1}{10^{-7}}$$

If $x = 1$ and $T = 298$ K, then,

$$\Delta G^0 = \Delta G^{0'} + 40.0 \text{ kJ}$$

This means that for reactions producing H^+ ions, $\Delta G^{0'}$ is greater than ΔG^0 by 40.0 kJ per mole of H^+ ions released. Hence the reaction is more spontaneous at pH 7 than at pH 0. On the other hand, if H^+ ion appears as the reactant,

$$C + xH^+ \longrightarrow A + B$$

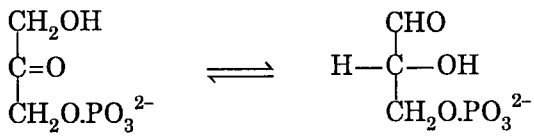
we can show that

$$\Delta G^0 = \Delta G^{0'} - 40.0 \text{ kJ}$$

and this reaction will be more spontaneous at pH 0 than at pH 7. For reactions not involving H^+ ions, ΔG^0 is equal to $\Delta G^{0'}$.

4.10 Standard Free Energy Changes at pH 7.0 or $\Delta G^{0'}$

In order to calculate $\Delta G^{0'}$ we will consider an example of isomerization of dihydroxy-acetonc phosphate (DHAP) to glyceraldehyde 3-phosphate (G-3-P). It has been one of the reaction of glycolysis.



Dihydroxyacetone phosphate Glyceraldehyde 3-phosphate

At equilibrium, the ratio of glyceraldehyde 3-phosphate to dilydroxy acetone phosphate has been found to be 0.0475 at 25°C (298°K) and pH 7.0. Hence,

$$K'_{eq} = 0.0475$$

The standard free energy change for this reaction is, the, calculated by using equation given as follows :

$$\Delta G^{0'} = -2.303\ RT \log_{10} K'_{eq} \quad \ldots(1)$$
$$= -2.303 \times 1.98 \times 10^{-3} \times 2.98 \times \log_{10} (0.0475)$$
$$= +1.8 \text{ kcal/mol.}$$

If the initial concentration of DHAP has been 2×10^{-4} M and the initial concentration of G-3-P is 3×10^{-6} M, then ΔG can be calculated by using equation given as follows :

$$\Delta G = \Delta G^0 + RT \log_e \frac{[C]\,[D]}{[A]\,[B]}$$

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$$= 1.8 \text{ kcal/mol} + 2.303\ RT \log_{10} \frac{3 \times 10^{-6} M}{2 \times 10^{-4} M}$$

$$= 1.8 \text{ kcal/mol} - 2.5 \text{ kcal/mol}$$

$$= -0.7 \text{ kcal/mol}$$

$$= -2.93 \text{ kJ/mol}$$

The negative value for ΔG reveals that isomerization of dihydroxyacetone phosphate to glyceraldehyde 3-phosphate can take place spontaneously, when these compounds are present at concentrations mentioned above. Although ΔG has been negative for this reaction, yet the value of $\Delta G^{0'}$ has been positive. It is important to note that the magnitude of ΔG for a reaction (whether smaller, larger or the same as $\Delta G^{0'}$) has been found to depend on the concentrations of the reactants. The criterion of spontaneity for a reaction is ΔG and not $\Delta G^{0'}$. Table 4.3 lists the standard free energy changes for some chemical reactions.

Table 4.3

STANDARD FREE ENERGY CHANGES FOR REPRESENTATIVE CHEMICAL REACTIONS UNDER STANDARD CONDITIONS (THAT IS, AT 25°C AND PH 7.0)

Reaction type and reactions	$\Delta G^{0'}$ *(in kcal/mol)*	$\Delta G^{0'}$ *(in kJ/mol)*
Elimination of water		
Malate → Fumarate + H_2O	+ 0.75	+ 3.138
Rearrangement		
Glucose 1-phosphate → Glucose 6-phosphate	– 1.74	– 7.280
Fructose 6-Phosphate → Glucose 6-phosphate	– 0.40	– 1.6736
Hydrolysis		
Esters		
Ethyl acetate + H_2O → Ethanol + Acetate	– 4.7	– 19.665
Glucose 6-phosphate + H_2O → Glucose + Phosphate	– 3.3	– 13.807
Amides and peptides		
Glutamine + H_2O → Glutamate + NH_4^+	– 3.4	– 14.225
Glycylglycine + H_2O → 2 Glycine	– 2.2	– 9.205
Glycosides		
Maltose + H_2O → 2 Glucose	– 3.7	– 15.481
Lactose H_2O → Glucose + Galactose	– 3.8	– 15.899
Acid anhydrides		
Acetic anhydride + H_2O → 2 Acetate	– 218	– 91.211
ATP + H_2O → ADP + Phosphate	– 7.3	– 30.543
Oxidations with molecular oxygen		
Glucose + $6O_2$ → $6CO_2$ + $6H_2O$	– 686	– 2870.224
Palmitic acid + $23O_2$ → $16CO_2$ + $16H_2O$	– 2338	– 9782.192

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When we go through the above table, we find that hydrolysis of esters, amides, peptides and glycosides, as well as rearrangements, and elimination takes place with relatively small standard free energy change. Hydrolysis of acid anhydrides takes place with relatively large decrease in standard free energy, whereas oxidation of organic compounds to CO_2 and H_2O occurs with rather huge decrease in standard free energy.

4.11 Difference Between ΔG and $\Delta G^{0'}$

It is better if we understand the difference between the free energy change, ΔG and the standard free energy change, $\Delta G^{0'}$ of a chemical reaction. The actual free energy change, ΔG of a chemical reaction has been a function of the conditions of concentration, *p*H and temperature under which the reaction is occurring. Moreover the ΔG of an ongoing chemical process always becomes negative, becomes smaller (*i.e.*, less negative) as the reaction proceeds and becomes zero at the point of equilibrium, revealing that no more work can be done by the reaction. The value of ΔG decreases with time as the reaction proceeds because the actual concentrations of the reactants will be getting smaller and those of the resultants getting larger.

Thus, it can be concluded that in every spontaneous chemical or physical process, the free energy of the reacting system always decreases, *i.e.*, ΔG is negative. On the other hand, the value of standard free energy change, $\Delta G^{0'}$ for a chemical reaction has been characteristic and unchanging, and may be positive, negative or zero, depending on the equilibrium constant of the reaction. $\Delta G^{0'}$ is, thus, an immutable constant and predicts in which direction and how far a given reaction will go in order to reach equilibrium when it takes place under standard conditions, *i.e.*, when the initial concentration of all components is 1.0 M, the pH is 7.0 and the temperature is 25°C.

4.12 Standard Free Energy Values of Chemical Reactions are Additive

An important thermodynamic fact is that the overall free energy change for a series of reactions has been equal to the sum of the free energy changes of the individual steps. Let us consider the two consecutive reactions where each reaction is having its own equilibrium constant and a characteristic standard free energy change, $\Delta G_1^{0'}$ and $\Delta G_2^{0'}$.

$$A \longrightarrow B \qquad \Delta G_1^{0'} = -8 \text{ kcal/mol}$$

$$B \longrightarrow C \qquad \Delta G_2^{0'} = +5 \text{ kcal/mol}$$

As the two reactions are sequential, the intermediate product B cancels out and the overall reaction with its standard free energy change, $\Delta G_s^{0'}$ my be put as follows :

$$A \longrightarrow C \qquad \Delta G_s^{0'} = \Delta G_1^{0'} + G_2^{0'}$$

$$= -8 + (+5)$$

$$= -3 \text{ kcal/mol}$$

$$= -12.552 \text{ kJ/mol}$$

Under standard conditions, A can get spontaneously converted into B as ΔG is negative. However, the conversion of B into C, under standard conditions has been not thermodynamically feasible. But as the free energy changes are additive, the conversion of A into C has a $\Delta G^{0'}$ value of – 12.552 kJ/mol, which obviously implies that A can be converted into C spontaneously under standard conditions. Thus, the above two sequential reactions get coupled by the intermediate product, B. In other words, *a thermodynamically unfavourable reaction can be driven by a thermodynamically favourable reaction.*

Two sequential steps from glycogen break down in muscles will demonstrate this fact more clearly.

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$$\text{Glucose 1-Phosphate} \xrightarrow{\text{Phosphogluco-mutase}} \text{Glucose 6-phosphate}, \quad \Delta G_1^{0'} = -1.74 \text{ kcal/mol}$$

$$\text{Glucose 6-phosphate} \xrightarrow{\text{Glucose phosphate isomerase}} \text{Fructose 6-phosphate} \quad \Delta G_2^{0'} = +0.40 \text{ kcal/mol}$$

On adding the two reactions, we obtain

$$\text{Glucose 1-phosphate} \longrightarrow \text{Fructose 6-phosphate}$$

This has a standard free energy change value,

$$\begin{aligned} \Delta G_s^{0'} &= \Delta G_1^{0'} + \Delta G_2^{0'} \\ &= -1.74 + (+0.40) \\ &= -1.36 \text{ kcal/mol} \\ &= -5.6902 \text{ kJ/mol} \end{aligned}$$

As $\Delta G_s^{0'}$ is negative, glucose 1-phosphate gets converted into fructose 6-phosphate in the muscles.

4.13 Coupled Reactions

In biochemical reactions, two or more reactions may be taking place together in such a way that an exergonic reaction may be used to drive an endergonic one. Such reactions are known as coupled reactions.

The simplest type of coupling (Fig. 4.3) may be represented by the equation :

$$A + C \longrightarrow B + D + \text{Heat}$$

The conversion of metabolite A to metabolite B takes place with the release of energy. It is coupled to another reaction, wherein energy is required to convert metabolite C to metabolite D.

Many instances of this sort of coupling are seen in metabolism when the energy derived from an exergonic reaction (usually an oxidation) is used to drive an endergonic synthetic reaction. This sort of coupling requires both reactants to contain a common obligatory intermediate.

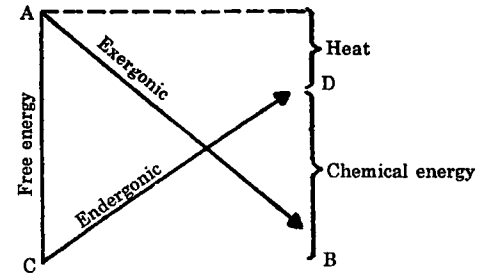

Fig. 4.3. Coupling of an exergonic to an endergonic reaction.

$$A + C \longrightarrow I \longrightarrow B + D$$

This type of coupling should have a built-in mechanism for biological control of the rate at which oxidative processes are allowed to take place because the existence of a common obligatory intermediate for both the exergonic and endergonic reactions allows the rate of utilisation of the product of the synthetic path (D) to determine by mass action the rate of which A is oxidised. This type of coupling is provided by dehydrogenation reactions which are coupled to hydrogenation by an intermediate carrier.

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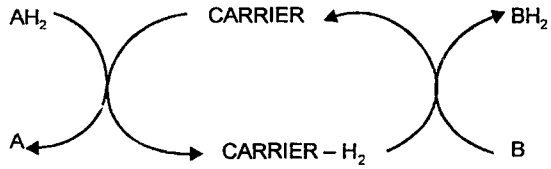

An alternative method of coupling an exergonic to an endergonic process may involve the synthesis of a new compound of high-energy potential in the exergonic reaction and then this new compound may be incorporated into endergonic reaction, thus effecting a transference of free energy from the exergonic to the endergonic pathway.

In Fig. 4.4. ~E is a compound of high potential energy and E is the corresponding compound of low potential energy.

The main advantage of this mechanism of coupling is that E unlike I in the previous mechanism may not be structurally related to A, B, C, or D. This would make E to serve as a transducer of energy from a wide range of exergonic reactions to an equally wide range of endergonic reactions or processes. In the living cell, the principal high-energy intermediate or carrier compound (designated ~E) is *adenosine triphosphate* or *ATP.*

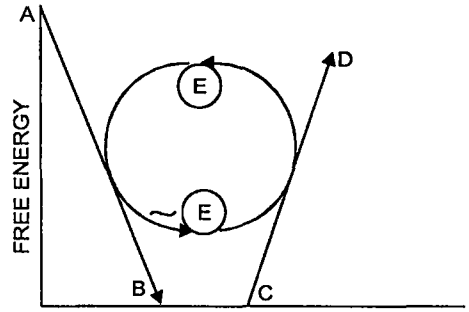

Fig. 4.4. Transference of free energy from an exergonic to an endergonic reaction through the formation of a high energy intermediate compound.

4.14 Energy Rich Compounds and Energy Coupling

Introduction : In order to maintain living processes, all organisms must get free energy from their environment. The autotrophic organisms get supply of free energy by coupling their metabolism to some simple exergonic process in their surroundings. For example, green plants utilise the energy of sunlight. On the other hand, heterotrophic organisms get free energy by coupling their metabolism to the breakdown of complex organic molecules in their environment.

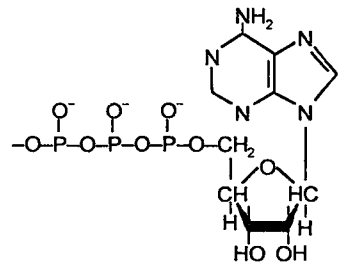

Fig. 4.5. Adenosine triphosphate (ATP)

The special carrier of free energy has been adenosine triphosphate (ATP). *ATP plays a central role in the transference of free energy from the exergonic (= energy yielding) to the endergonic (= energy requiring) processes in the cells.* During breakdown of energy rich foodstuffs or fuel molecules, some of the free energy gets harnessed to make ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i), a process that requires input of free energy. ATP then donates much of its chemical energy to energy needing processes (biosyntheses, transport etc.) by undergoing a breakdown to ADP and P_i.

A large number of coupled reactions involved ATP or another nucleoside triphosphate. ATP is a specialised nucleotide having adenine, ribose and 3 phosphate groups (Fig. 4.5). In its reactions in the cell, it functions as the Mg^{+2} complex (Fig. 4.6).

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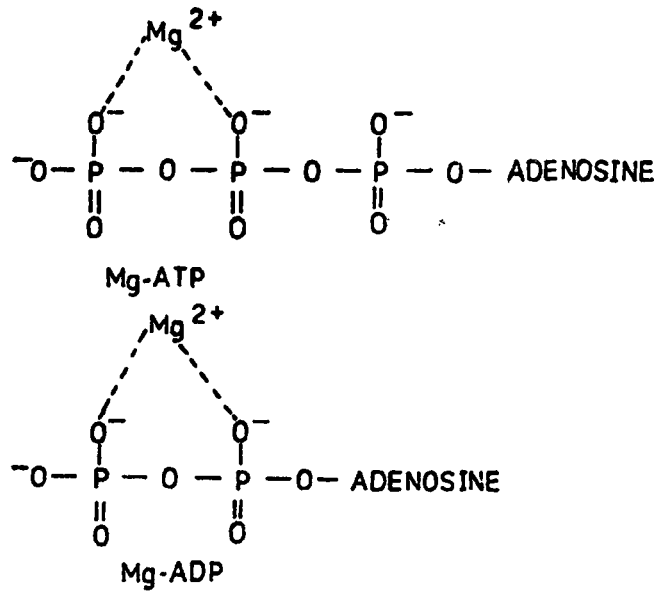
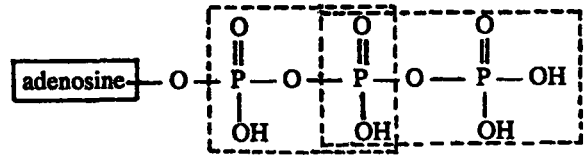


Fig. 4.6. The magnesium complexes of ATP and ADP.

The importance of phosphates in the intermediary metabolisms was realised by Fritz in 1941 and he introduced the concept of "high-energy phosphates" and "high-energy phosphate bonds."

High-Energy Phosphates

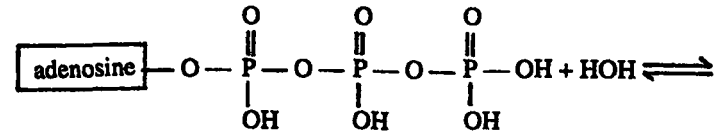
ATP is frequently described as *an energy-rich compound* with bonds called *high-energy bonds. A high energy bond is one that liberates a large amount of energy when hydrolyzed (> 5 kcal/mole).* Two such high-energy groups exist in the ATP anhydride linkage. Let's use a block diagram of ATP so that we can focus our attention on its high-energy groups.

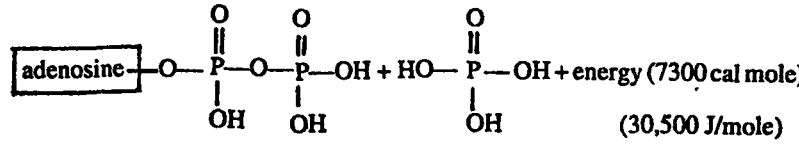


The anyhydrides can be hydrolyzed. Upon hydrolysis of 1 mole of ATP to adenosine diphosphate (ADP), about 7300 cal (30,500 J) are liberated under the operating conditions of the cell.

Hydrolysis of ATP to ADP

Adenosine triphosphate (ATP) + water $\rightleftharpoons$ adenosine diphosphate (ADP) + P_i + energy





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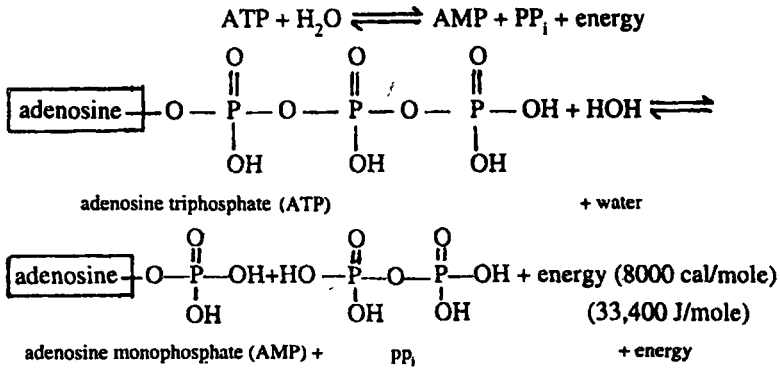
P_i indicates *inorganic phosphate,* all forms of phosphate not combined with a carbon compound.

ATP can also hydrolyze exothermically to produce adenosine monophosphate (AMP) and pyrophosphate (PP_i)

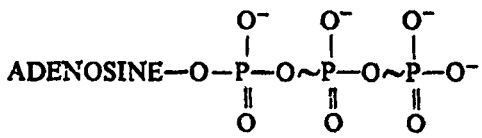


Thus, by exothermically hydrolyzing ATP to ADP or AMP, a cell liberates energy which can be used to power endothermic processes.

The hydrolysis of ADP to AMP is also exothermic but does not occur frequently in cellular metabolism.

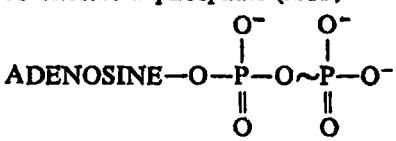
In order to indicate the presence of the high energy phosphate group, Lipman employed the symbol NP to indicate *high-energy phosphate bond.*

This symbol is indicative of the fact that the group attached to the bond, when transferred to an appropriate acceptor, results in transfer of the larger quantity of energy. Due to this reason the *group transfer potential* is used to indicate *"high-energy bond."* For instance, ATP has two high energy phosphate groups and ADP has one while the phosphate bond in AMP (adenosine monophosphate) is of the low-energy type because it is a normal ester link.



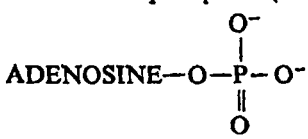
or ADENOSINE P~P~P

Adenosine triphosphate (ATP)



or ADENOSINE—P~P

Adenosine diphosphate (ADP)



Adenosine monophosphate (AMP)

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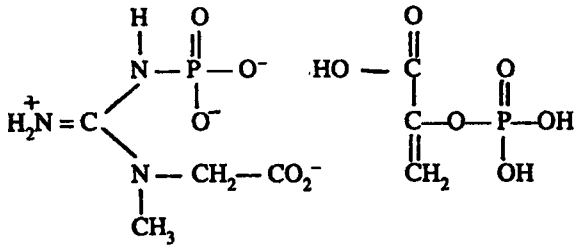
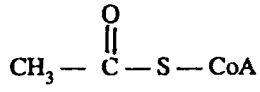
Cells also contain other high-energy compounds in addition to ATP. When hydrolyzed, these compounds also liberate more than 5 kcal/mole. Structural formulas for several of these compounds are given below.

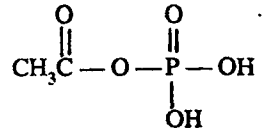


phosphocreatine
(creatine phosphate, found in muscle cells)
(10.3 kcal/mole)
(43.1 kJ/mole)

phosphoenol pyruvic acid (PEP)
(14.8 kcal/mole)
(61.9 kJ/mole)

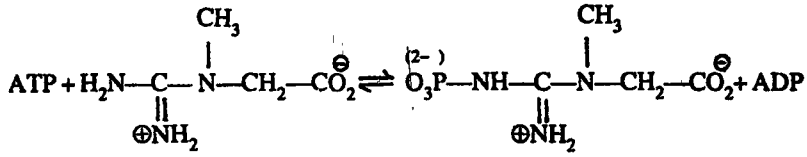


acetylcoenzyme A
(8 kcal/mole)
(33 kJ/mole)



acetyl phosphate
(10.5 kcal/mole)
(43.9 kJ/mole)

Phosphocreatine (creatine phosphate) is an important phosphate storage compound. The phosphate group is added directly and reversibly to creatine from ATP under the influence of the enzyme creatine kinase.



creatine phosphocreatine

These reversible reactions are particularly important in skeletal muscle tissue. During muscular work, ATP is needed for muscle contraction. When the muscle is at rest, ATP concentration is high and the forward reaction above is favoured, a phosphate group is transferred to creatine, forming phosphocreatine. In this way, creatine stores phosphate. During muscular work, ATP concentration decreases as ATP is converted to ADP. The increased ADP concentration causes the reverse reaction above to be favoured; direct phosphorylation of ADP by phosphocreatine occurs.

The hydrolysis of 1 mole of phosphocreatine liberates 10,300 cal (43,100 Joules).

$$\text{Phosphocreatine + water} \rightleftharpoons \text{creatine + phosphate + 10,300 cal}$$

Unlike the acid anhydride linkage hydrolysed in ATP, the P—N bond hydrolysed in phosphocreatine is an amide linkage.

Free energy of hydrolysis of ATP and other organophosphates : The standard free energy of hydrolysis of a number of biochemically important phosphates has been given in Table 4.4.

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Table 4.4
STANDARD FREE ENERGY OF HYDROLYSIS OF SOME ORGANOPHOSPHATES OF BIOCHEMICAL IMPORTANCE

Compound	$\Delta G^{0'}$	
	kJmol^{-1}	*kcal/mole*
(*i*) Phosphoenol pyruvate	– 61.1	– 14.8
(*ii*) Carbamoyl Phosphate	– 51.4	– 12.3
(*iii*) 1,3-Biphosphoglycerate-(to 3-phosphoglycerate)	– 49.3	– 11.8
(*iv*) Creatine phosphate	– 13.1	– 10.3
ATP ⟶ ADP + P*i*	– 36.8	– 8.8
(*v*) Glucose 1-phosphate	– 20.9	– 5.0
(*vi*) Fluctose 6-phosphate	– 15.9	– 3.8
(*vii*) Glucose 6-phosphate	– 13.8	– 3.2
(*viii*) Glycerol 3-phospate	– 9.2	– 2.2

From Table 4.4 it can be seen that the value for the hydrolysis of the terminal phosphate of the ATP of – 36.8 kJ (– 8.8 kcal) per mol (as it is also for the terminal phosphate of ATP) divides the list into 2 groups.

(i) Low energy compounds : This group has $\Delta G^{0'}$ values which are smaller than that of ATP. This group is exemplified by the ester phosphates found in the intermediates of glycolysis.

(ii) High energy compounds : This group has $\Delta G^{0'}$ values which are higher than that of ATP. This group is exemplified by anhydrides (*e.g.*, ATP, ADT, the 1-phosphate of 1, 3-biphosphorycreate), enolphosphates (*e.g.*, phosphenol-pyruvate) and phosphogunidines (*e.g.*, creatine phosphate, arginine phosphate). Other biologically important compounds that are classified as "high-energy compounds" are thiol esters involving coenzyme. A (*e.g.*, acetyl CoA), acyl carrier protein, amino acid esters involved in protein synthesis, S-adenosylmethionine (active methionine) and UDPG (uridine diphosphate glucose).

Role of high energy phosphates as the energy currency of the cells : Due to its middle position in the Table 4.4, ATP acts as a donor of high-energy phosphate to those compounds which are below it in the Table 4.4. Similarly, ADP can accept high-energy phosphate to form ATP from those compounds which are above ATP in the Table 4.4. In reality, an ATP/ADP cycle connects these processes which generate ~ P to those processes which utilise ~P.

In Table 4.4, there is another group of compounds which act as storage forms of high-energy phosphate. These are creatine phosphate (phosphagen) and arginine phosphate.

Bioenergetics of coupled reactions : In biological systems, ATP serves as a source of energy for many of the endergonic reactions. Also, ATP get synthesised in many exergonic reactions.

When an energonic reaction gets coupled with an exergonic reaction through ATP, the overall effect of this coupling involves the transfer of kinetic energy from one system to another. Several enzymes are known which facilitate these coupling reactions. The coupling of reactions so as to bring about efficient transfer of energy is quite common in biological systems. This is illustrated by considering a reaction which is the first in the glycolysis pathway. For example, the formation

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of glucose 6-phosphate from glucose and phosphoric acid requires energy with $\Delta G^0 = +13.8$ kJ mol^{-1}.

(i) Glucose + $P_i \longrightarrow$ Glucose 6-P + H_2O; $\Delta G^{0'} = +13.8$ kJ mol^{-1}

The above reaction would not proceed as such under physiologic condition. In order to take place, the reaction must be coupled with another reaction which should be more exergonic than the phosphorylation of glucose which is endergonic. Such a reaction is nothing but the hydrolysis of the terminal phosphate of ATP.

(ii) ATP $\longrightarrow$ ADP + P_i; $\Delta G^{0'} = -36.8$ kJ mol^{-1}

If (*i*) and (*ii*) get coupled in a reaction catalysed by hexokinase, there occurs ready phosphorylation of glucose in a highly exergonic reaction that under physiologic conditions is far from equilibrium and thus irreversible for practical purposes.

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose 6.P} + \text{ADP}$$
$$\Delta G^{0'} = +23.2 \text{ kJ mol}^{-1}$$

Many "activation reactions" have been found to follow this pattern.

This series of coupled reactions may be put as follows :

By using energy coupling a complex compound could be synthesized apparently which violates the second law of thermodynamics. But the algebraic sum of energy of all the components of coupled reaction is negative, *i.e.*, the sum of energy liberated has been found to be more than the sum of energy consumed. In biological systems, the hydrolysis of energy rich compounds is generally accompanied by an endergonic reactions so that the energy liberated in the former reaction is used fully without getting wasted. This type of control needs numerous enzymes which are present in the cell. The high energy compounds get dissociated only in the presence of enzymes.

Adenylate Kinase Allows Interconversion of Adenine Nucleotides

The enzyme adenylate kinase (myokinase) is present in most cells. It catalyzes the interconversion of ATP and AMP on the one hand and ADP on the other :

$$\text{ATP} + \text{AMP} \xleftarrow{\boxed{\text{ADENYLATE KINASE}}} 2\text{ADP}$$

This reaction has 3 functions :

(1) It allows high-energy phosphate in ADP to be used in the synthesis of ATP.

(2) It allows AMP, formed as a consequence of several activating reactions involving ATP, to be recovered by rephosphorylation to ADP.

(3) It allows AMP to increase in concentration when ATP becomes depleted and act as a metabolic (allosteric) signal to increase the rate of catabolic reactions, which in turn leads to the generation of more ATP.

When ATP Reacts to Form AMP, Inorganic Pyrophosphate (PP_i) is Formed

This occurs, for example, in the activation of long-chain fatty acids.

$$\text{ATP} + \text{CoA.SH} + \text{R.COOH} \xrightarrow{\boxed{\text{ACYl-CoA SYNTHETASE}}} \text{AMP} + PP_i + \text{R.CO} - \text{SCoA}$$

This reaction is accompanied by loss of free energy as heat, which ensures that the activation reaction will go to the right; this is further aided by the hydrolytic splitting of PP_1, catalysed by

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inorganic pyrophosphatase, a reaction that itself has a large $\Delta G^{0'}$ of – 27.6 kJ/mol. Note that activations via the pyrophosphate pathway result in the loss of 2 ~ P rather than one ~ P as occurs when ADP and P_i are formed.

A combination of the above reactions makes it possible for phosphate to be recycled and the adenine nucleotides to interchange (Fig. 4.7).

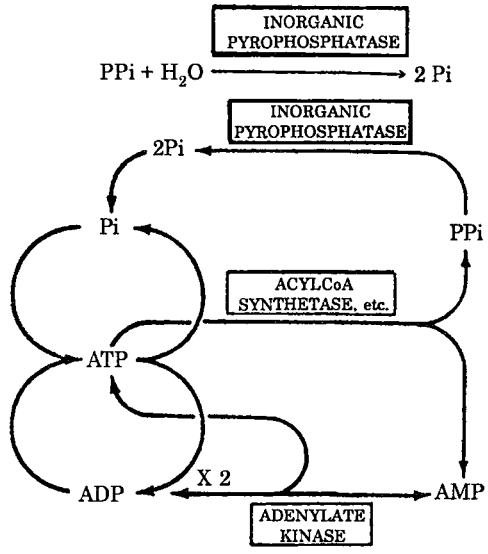

Fig. 4.7. Phosphate cycles and interchange of adenine nucleotides.

ATP synthesis : Respiratory Chain of Events

Energy is released upon ATP hydrolysis. This means that the reverse process is endothermic and energy is stored in ATP during its formation. (Fig. 4.8).

$$7300 \text{ cal} + \text{ADP} + Pi \rightleftharpoons \text{ATP} + \text{HOH}$$

$$8000 \text{ cal} + \text{AMP} + PPi \rightleftharpoons \text{ATP} + \text{HOH}$$

A logical question is : Where does the energy come from that's used in ATP formation ? It arises during catabolism in which cells chemically break down large metabolite molecules into simpler substances of *lower* energy. A net release of energy occurs and some of the energy is stored by conversion of AMP and ADP to ATP. The formation of ATP by capturing energy liberated during catabolism is called *oxidative phosphorylation.* The majority of ATP is produced by this process. Oxidative phosphorylation occurs through the action of the *respiratory chain,* also known as the *cytochrome chain or the electron transport system* (ETS). The components of the ETS are found on the inner membrane surface of mitochondria.

The enzymes of the ETS function as a sequence in which hydrogens are passed along carriers. Ultimately, the hydrogens combine with oxygen to produce water.

Hydrogen atoms can be imagined to consist of component hydrogen ions and electron.

$$H + H = 2e + 2H^+$$

Recall that the loss of hydrogen with its associated electron from a substance is called *oxidation;* gain of hydrogen and its electron is called *reduction.* The formation of water involves the reaction

$$2H^+ + 2e^- + 1/2\ O_2 \longrightarrow H_2O$$

Therefore, a biological requirement exists for hydrogen ion and electron carriers.

Two kinds of carriers are present in the electron transport system : (1) coenzymes, which are electron and hydrogen ion carriers, and (2) cytochromes, which are exclusively electron carriers. The coenzymes presently known to participate in the ETS are (1) nicotinamide adenine dinucleotides (NAD and NADP), (2) flavin nucleotides (FAD and FMN), and (3) coenzyme Q (CoQ). (Fig. 4.8).

High-Energy Phosphates Play a Central Role in Energy Capture and Transfer

In order to maintain living processes, all organisms must obtain supplies of free energy from their environment. *Autotrophic* organisms couple their metabolism to some simple exergonic process in their surroundings, *e.g.,* green plants utilise the energy of sunlight, and some

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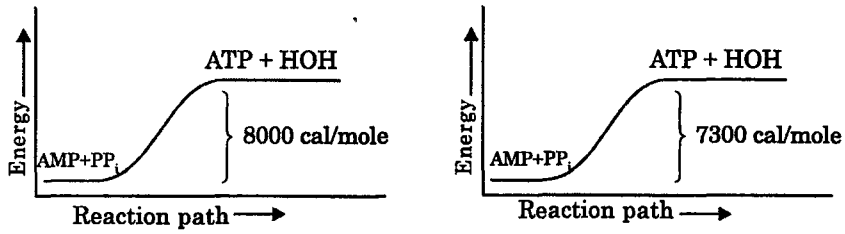

Fig. 4.8. ATP formation as an endothermic process.

autotrophic bacteria utilise the reaction $Fe^{2+} \longrightarrow Fe^{3+}$. On the other hand, *heterotrophic* organisms obtain free energy by coupling their metabolism to the breakdown of complex organic molecules in their environment. In all of these processes, ATP plays a central role in the transference of free energy from the exergonic to the endergonic processes (Fig. 4.9).

The importance of phosphates in intermediary metabolism became evident with the discovery of the chemical details of glycolysis and of the role of ATP, adenosine diphosphate (ADP), and inorganic phosphate (P_i) in this process. ATP was considered a means of transferring phosphate radicals in the process of phosphorylation. The role of ATP in biochemical energetics was indicated in experiments demonstrating that ATP and creatine phosphate were broken down during muscular contraction and that their resynthesis depended on supplying energy from oxidative processes in the muscle. It was not until Lipmann introduced the concept of "high-energy phosphates" and the "high-energy phosphate bond" that the role of these compounds in bioenergetics was clearly appreciated.

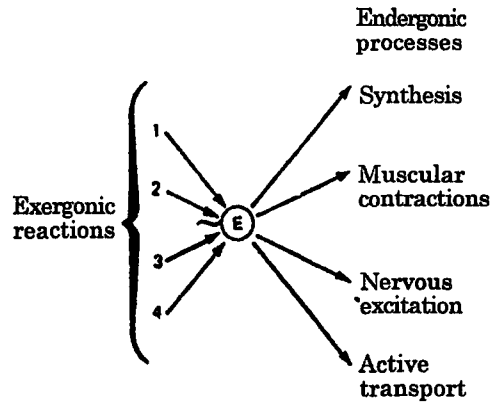

Fig. 4.9. Transaction of energy through a common high-energy compound to energy-requiring

Mechanism of ATP Synthesis

The synthesis of ATP from ADP and inorganic phosphate, P_i takes place on mitochondrial and chlorophastic membranes. During electron transport in the oxidation of NADH in mitochondria or in transfer of electrons from water to $NADP^+$ in illuminated chloroplast, there occurs liberation of energy according to the difference in redox potentials of electron donating and electron receiving components. A part of this energy is consumed in the synthesis of high energy ATP molecules from ADP and P_i. This synthesis takes place on the inner surface of the membranes.

There are three hypotheses which have been postulated to explain the mechanism of ATP synthesis.

I. Chemical hypothesis : This hypothesis was postulated by E.C. Slater (1953) to explain mainly the substrate phosphorylation. According to this hypothesis, there are some definite chemical compounds which participate in phosphorylation. The energy of electron transfer from one substrate to the other is first of all taken up by these compounds to form energised compounds. Then, these energised compounds activate inorganic phosphate which combines with ADP to form ATP. The sequence of these processes may be depicted as follows :

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$$AH_2 + B + I \rightleftharpoons A \sim I + BH_2$$

$$A \sim I + X \rightleftharpoons A + X \sim I$$

$$X \sim I + Pi \rightleftharpoons X \sim Pi + I$$

$$X \sim Pi + ADP \rightleftharpoons X + ATP$$

where AH_2 and B are two substrates and X and I are hypothetical intermediates.

II. Conformational hypothesis : The conformational hypothesis of ATP synthesis was postulated by P.D. Boyer (1967). According to this hypothesis, the energy of electron transport is accepted by macromolecule or a portion of the membrane, causing a conformational change in the macromolecule or membrane. Then the activated macromolecule with changed configuration relaxes releasing the energy for carrying out ATP synthesis (Fig. 4.10).

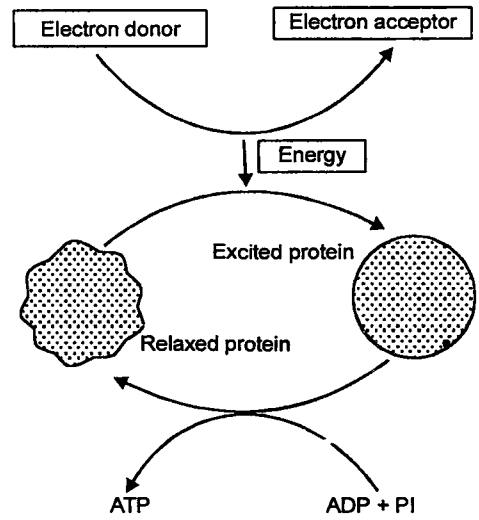

Fig. 4.10. Involvement of a structural protein in ATP synthesis (conformational hypothesis).

A modification of the model given by Green and Ji, 1972 suggests that conformational change is induced by an electric field which is developed by the electron transport chain.

III. Chemi-osmotic hypothesis : This hypothesis was given by Peter Mitchele (1961, 1968). According to this hypothesis, the electron transport can be coupled to phosphorylation of ADP involving a chemi-osmotic process. This hypothesis has been equally applicable to the photo-phosphorylation in the chloroplast as well as the oxidative phosphorylation in the mitochondria.

This hypothesis is assumed to involve both enzyme ATPase and a well organised membrane.

$$ADP + P_i \overset{\text{ATPase}}{\rightleftharpoons} ATP + H_2O$$

In the reversible reaction, removal of water from active center of ATPase will tend to favour synthesis of ATP in accordance to law of mass action. This assumption forms the fundamental basis of Mitchell's chemi-osmotic hypothesis. In the later modifications of the hypothesis, involvement of chemical intermediates X and I has also been postulated. The details of mechanism have been given in Fig. 4.11.

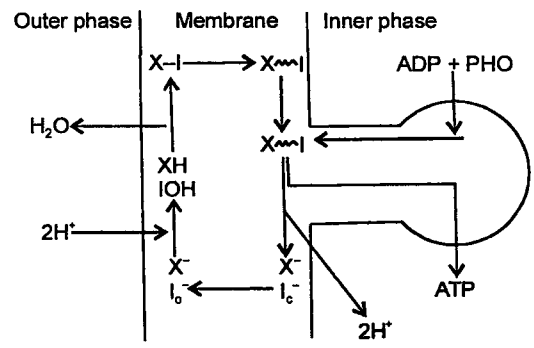

Fig. 4.11. Mitchell's modified scheme of chemiosmotic hypothesis.

The electron transport in the membrane involves two types of effects related to ATP synthesis. (*i*) Ionisation of water and separation of H^+ and OH^- and (*ii*) formation

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of high energy X~I complex from two unknown chemical intermediates X and I. Positively charged hydrogen ions undergo combination with the complex X^-IO^- on the outer surface of the membrane and H_2O gets liberated. Later on X and I combine to form a complex which gets converted to a high energy X~I by the energy released during electron transport, as described above. This X~I moves towards the innerside of the membrane where it undergoes combinations with ADP, P_i and OH^- to yield ATP and releases H^+ on the innerside of the membrane. The complex X^-10^- moves again to the outer side of the membrane to combine with the hydrogen ion and the process continues further, producing one ATP molecule in each cycle on the inner surface of the membrane. Under conditions, where ATP synthesis does not take place, the ejection of proton H^+ is balanced by uptake of Ca^{++}. For every pair of proton one Ca^{++} gets absorbed inside.

Chemiosmotic hypothesis also needs the involvement of two chemical intermediates (of unknown chemistry), as it happens in case of chemical hypothesis. In chemiosmotic hypothesis, however, these chemical intermediates do not require to enter into combination with an electron carrier in the respiratory chain. Further, the propounders of chemiosmotic hypothesis have been much more flexible about the chemical nature of X and I than those of chemical hypothesis, who was able to predict that these are discrete covalent compounds.

Chemiosmotic hypothesis is known to accommodate most of the experimental observations. The production of pH gradient across membrane, as predicted by this hypothesis, is frequently observed during phosphorylation. The pH of the medium having actively phosphorylating isolated mitochondria decreases. This decrease can be stopped by adding antimycin, an uncoupler of phosphorylation. Further, synthesis of ATP in isolated mitochondria from rat liver or beet roots gets simulated by lowering the pH of the medium. This stimulation does not take place in the presence of dinitrophenol or oligomycin.

Peter Mitchell was awarded Nobel Prize for his theory.

Redox Potential

When NADH is oxidised in the mitochondria to produce ATP, there occurs transfer of electrons from one substrate to the other. This flow of electrons takes place according to the relative affinities of the substrate to gain or lose electrons. This process of electron transfer is accompanied with the oxidation-reduction of the system. A compound losing electron gets oxidised whereas a compound gaining it gets reduced. For example, ferrous ion is oxidised to ferric ion by losing one electron and *vice versa*. The quantitative measure of the affinity of a compound to lose or gain electrons is termed as the redox potential (reduction-oxidation potential).

$$Fe^{2+} \underset{\text{reduction}}{\overset{\text{oxidation}}{\rightleftharpoons}} Fe^{3+} + e$$

Redox potential can be measured through a standard hydrogen electrode which has an arbitrary electrode potential of zero at 1 N concentration and 1 atmosphere pressure. In a similar manner, the electrode potential of a reducing-oxidising system can also be measured. In this case electrode potential will be the redox potential.

It is possible to determine redox potential by using Peter's equation.

$$\text{Redox potential (E)} = E_0 + \frac{RT}{nF} \ln \frac{[\text{oxidant}]}{[\text{reductant}]}$$

where E_0 is the redox potential of mixture containing equimolal concentrations of the oxidant and the reductant, R is gas constant in joules per mole per degree, T is the absolute temperature, F is Faraday number (96,500 coulombs), n is the number of electrons per g equivalent of reactant and ln is the logarithm to the base e.

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Redox System and Free Energy

In a redox system, there occurs flow of electrons. This generates electricity which is used to perform work. The difference in the redox potential of two systems determines the degree of electron flow or in other words the change in free energy. The change in free energy in a redox system can be calculated by using the following equation :

$$\Delta G = -nF\,\Delta E_0$$

where n denotes the number of electrons transferred, F denotes Faradey number (96,500 coulombs) and ΔE_0 denotes the difference in redox potential.

The oxidation of NADH in the mitochondria is the most important energy releasing biological redox system. During this process there occurs transfer of electrons from NADH to molecular oxygen through cytochromes according to their redox potentials. The NADH is having a strong affinity to lose electrons while oxygen can accept them. Each individual step in the process is associated with its own ΔE which can be calculated from the difference in redox potential. The free energy required for the synthesis of one ATP molecule is about 29 kcal/mole. Three ATP molecules get synthesized during complete oxidation of NADH at three steps which possess large E_0 to produce enough energy for the chemical reaction. When we take the whole series as one reaction, ΔE from NADH to molecular oxygen is 1.13. The free energy with this value of ΔE will be $2 \times 96.5 \times 1.13 = 208$ kcal according to the equation. Synthesis of three molecules of ATP will require $29 \times 3 = 87$ kcal of energy. Hence, about 42% of the total energy produced during this process is conserved in the chemical synthesis of ATP. This is quite an efficient system as far as conservation of energy is concerned. Rest of the energy would probably escape as heat.

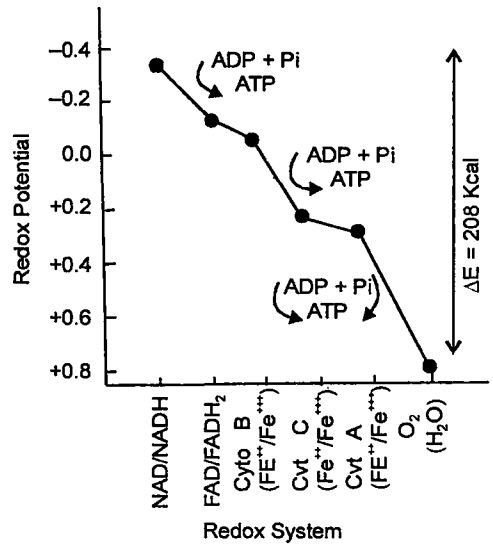

Fig. 4.12. Redox potential and generation of ATP in the electron transport system.

4.15 Limitations of Thermodynamics to Biochemical Processes

So far we have presented a rather general and qualitative description of how some of the thermodynamic concepts can be applied to help us understand the nature of biochemical reactions. In this section we consider a more fundamental question : Under what conditions is thermodynamics applicable to biology ? The answer to this question is not a trivial one, and at present there is much debate on this subject.

To begin with, we note that biochemical reactions are usually discussed in terms of standard free energies (ΔG^0). Strictly, the direction of a reaction such as

$$A + B \rightleftharpoons C + D$$

is given by ΔG, where

$$\Delta G = \Delta G^0 + RT \ln \frac{[C]\,[D]}{[A]\,[B]}$$

Only in the case where the reactants and products are in their standard states we can employ ΔG^0. Thus the fact that the hydrolysis of ATP at 298 K and pH 7 results in a decrease of the

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standard free energy equal to – 30.5 kJ mol^{-1} does not necessarily mean that this is also the value for ΔG. In living cells, the physiological temperature, pH, concentration of reactants and products, as well as metal ions can vary from system to system, and these factors must affect the value of ΔG. It is difficult and in some cases impossible to accurately measure the concentration of various species in solution. Nevertheless, reasonable estimates can be made of the concentrations in many cases and hence of the sign of ΔG.

Another point that has often been raised is that thermodynamics deals only with closed systems at equilibrium. Living systems are open systems and they are maintained at steady state rather than at equilibrium. In fact, any cell at true equilibrium is dead cell. The treatment of open systems requires *irreversible thermodynamics.*

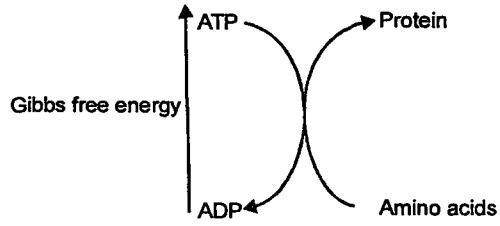

Fig. 4.13. Schematic representation of the Gibbs free energy changes that occur in protein synthesis.

Finally, reactions in a living cell, like any other reactions, can be classified into two categories—those that are thermodynamically controlled and those that are kinetically controlled. As an example of the former, let us consider the synthesis of a dipeptide from two amino acids :

$$\text{alanine} + \text{glycine} \longrightarrow \text{alanylgycine} \qquad \Delta G^0 = 17.2 \text{ kJ}$$

The corresponding equilibrium constant for this reaction is about 1×10^{-3} at 298 K. Clearly, such a process will not proceed by an appreciable extent if left on its own. However, if this process could be coupled to the hydrolysis of ATP by the action of an enzyme, the reaction would proceed from left to right (Figure 4.13). Such a reaction is said to be thermodynamically controlled because the reaction itself is not spontaneous, and the energy (or free energy) of reaction must be supplied from an outside source.

A kinetically controlled reaction is one in which the overall ΔG is negative (and hence thermodynamically favourable), but the rate is negligibly small in the absence of appropriate enzyme catalysis. The phosphorylation of glucose to glucose 6-phosphte (coupled to ATP hydrolysis) is certainly an exergonic process, but the reaction occurs at a very slow rate in the absence of the enzyme hexokinase.

4.16 Homeostasis

In living cells, very few individual reactions ever reach equilibrium because these reactions are often coupled with each other. This places a continuous stress preventing equilibrium from being reached. You would be dead *if all* cells in your body achieved equilibrium.

Consider that your body temperature serves as a useful guide to your physiological condition. The normal 98.6°F value and its relative constancy results from the *net* metabolic changes in your body even though your physical activities are not constant. You eat, sleep, work, walk, sit, sing—all different activities—and somehow your body accommodates these diverse functions without a drastic change in body temperature.

A prominent American physiologist Walter B. Cannon commented on this in 1933 :

The constant conditions which are maintained in the body might be termed *equilibria.* That word, however, has come to have fairly exact meaning as applied to relatively simple

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physicochemical states, in closed systems where known forces are balanced. The coordinated physiological processes which maintain most of the steady states in the organism are so complex and so peculiar to living beings involving, as they may, the brain and nerves, the heart, lung, kidneys and spleen, all working cooperatively that I have suggested a special designation for these states : *homeostasis*. The word does not imply something set immobile, stagnation. It means a condition—a condition which may vary, but which is *relatively* constant.

Thus, homeostasis involves a total system whose internal environment is constantly shifting materials from site to site in response to stresses placed upon it so that no *net* change in the environment occurs.

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5 Biochemical Techniques

5.1 Introduction

Experimental techniques have contributed substantially to the growth of modern biochemistry. Some of the techniques employed in biochemical research have been collected in this chapter. This will help the students to acquaint themselves with the terms and methods of practising biochemist.

5.2 Analytical Centrifugation and Cell Fraction

This technique is used to isolate and purify cellular organelles so that their structure and function can be studied in detail.

Centrifugation separation techniques are based upon the behaviour of particles in an applied centrifugal field. The particles are normally suspended in a specific liquid medium, held in tubes or bottles, which are located in a rotor. The rotor is positioned centrally on the drive shaft of the centrifuge. Particles which differ in density, shape or size can be separated since they sediment at different rates in the centrifugal field, each particle sedimenting at a rate which is proportional to the applied centrifugal field.

Centrifugation techniques are of two main types. *Preparative centrifugation* techniques are concerned with the actual separation, isolation and purification of, for example, whole cells, subcellular organelles, plasma membranes, polysomes, ribosomes, chromatin, nucleic acids, lipoproteins and viruses. For subsequent biochemical investigations, very large amounts of

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material may be involved when harvesting microbial cells from culture media, plant and animal cells from tissue culture or plasma from blood. Relatively large amounts of cellular particles may also be isolated in order to study their morphology, composition and biological activity. It is also possible to isolate biological macromolecules, such as nucleic acids and proteins, from preparations which have received some preliminary purification by, for example, fractional precipitation. In contrast, *analytical centrifugation* techniques are devoted mainly to the study of pure or virtually pure macromolecules or particles. It is primarily concerned with the study of the sedimentation characteristics of biological macromolecules and molecular structures, rather than with the actual collection of particular fractions. It requires only small amounts of material and utilises specially designed rotors and detector systems to continuously monitor the process of sedimentation of the material in the centrifugal field. Such studies yield information from which the purity, molecular weight and shape of the material may be deduced. Since preparative centrifugation techniques are more commonly used in undergraduate studies, this chapter will concentrate on these techniques and deal only briefly with analytical centrifugation techniques.

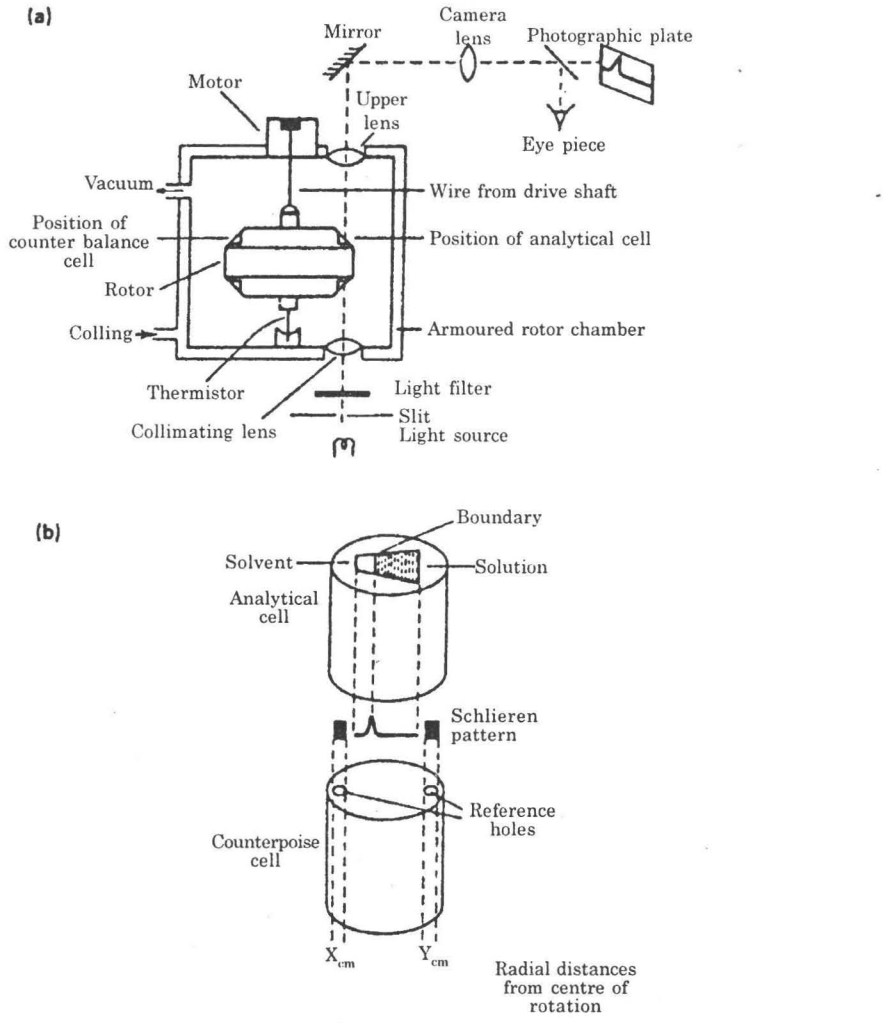

Fig. 5.1. Diagrammatic representation of (*a*) an analytical ultracentrifuge system and (*b*) an analytical and counterpoise cell.

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Analytical ultracentrifuges are capable of operating at speeds approaching 70,000 rev min^{-1} (500,000 *g*) and consist [Fig. 5.1 (*a*)] of a motor, a rotor contained in a protective armoured chamber which is refrigerated and evacuated and an optical system which enables the sedimenting material to be observed so as to determine concentration distributions within the sample at any time during centrifugation.

Ultracentrifuges invariably work under refrigeration and a partial vacuum to prevent air friction cooking the cell components. They can be used to separate the *molecular* components of cells by a technique called *density gradient centrifugation.* This involves suspending disrupted cells in a solution of similar *specific gravity,* such as *sucrose* or *caesium chloride solution,* and using carefully-chosen centrifugation times and speeds.

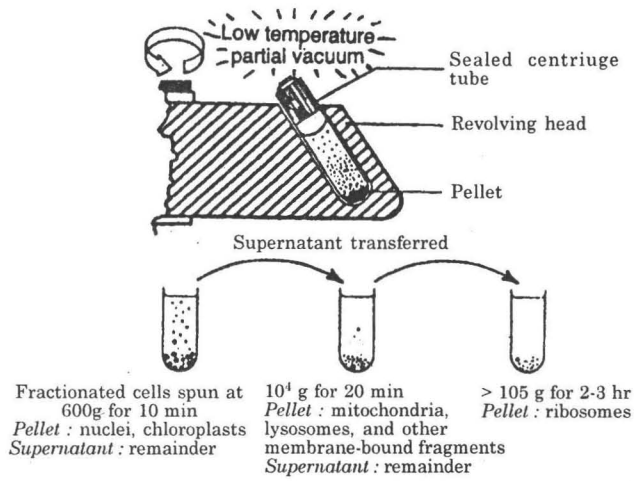

Fig. 5.2. Analytical ultracentrifugation.

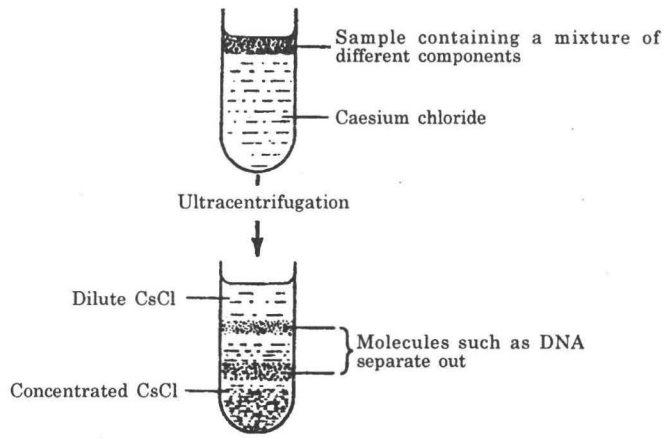

Fig. 5.3. A caesium chloride gradient.

In advanced analytical ultracentrifuges, the photographic plate detector system is replaced by an accurate electronic scanning system which can plot the concentration of the sample at all points in the analytical cell at any particular time. Different wavelengths of light can be selected enabling the separate movement of single components in a mixture of substances to be monitored provided they absorb light at different wavelengths.

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Subcellular Fractionation

In order to study macromolecules and metabolic processes within cells it becomes essential to isolate one type of *subcellular organelle* from the rest of the cell contents by subcellular fractionation. Initially, the plasma membrane (and cell wall if present) has to be ruptured. In order to do this, the tissue or cell sample is suspended in an isotonic sucrose solution (0.25—0.32 M) buffered at the appropriate pH, and the cells are then broken open by *homogenization* in a blender or homogenizer, by *sonication* or by subjecting them to high pressures (French press or nitrogen bomb). The initial homogenization, and the following subcellular fractionation, are usually performed at 4°C so as to minimize enzymic degradation of the cell's constituents. The sample of broken cells is often strained muslin or other fine gauze for removing larger lumps of material before proceeding further.

1. Differential velocity centrifugation : In differential velocity centrifugation, the various subcellular organelles are separated from one another on the basis of their *size*. A *centrifuge* is used to produce powerful forces; up to 100,000 times the force of gravity (*g*). The homogenized sample is kept in an appropriate centrifuge tube which is then loaded in the *rotor* of the centrifuge and subjected to centrifugation [Fig. 5.4 (*a*)]. Initially relatively low *g* forces are used for short periods of time but then increasingly higher *g* forces are used for longer time periods. For example, centrifugation at 600 *g* for 3 min would pellet the *nuclei*, the largest organelles [Fig. 5.4 (*b*)]. The supernatant from this step is removed to a fresh tube and then centrifuged at 6000 *g* for 8 min to pellet out *mitochondria, peroxisomes* and, if present, *lysosomes* or *chloroplasts*. Centrifugation of this next supernatant at 40000 *g* for 30 min will pellet out the *plasma membrane*, and fragments of the *endoplasmic reticulum* and *Golgi apparatus*. A final centrifugation at 100,000 *g* for 90 min would give rise to a *ribosomal pellet* and a supernatant that is essentially free of particulate matter and is considered to be the true soluble *cytosolic fraction*. However, the fractions isolated by differential velocity centrifugation are not usually entirely free of other subcellular organelles and so may need to be purified further. For separations at low *g* forces, a preparative centrifuge is used which has a rotor spinning in air at ambient pressure. However, an ultracentrifuge is needed for separations at higher *g* forces. The chamber of the ultracentrifuge is kept in a high vacuum to reduce friction, and subsequent heating, which would otherwise take place between the spinning rotor and air.

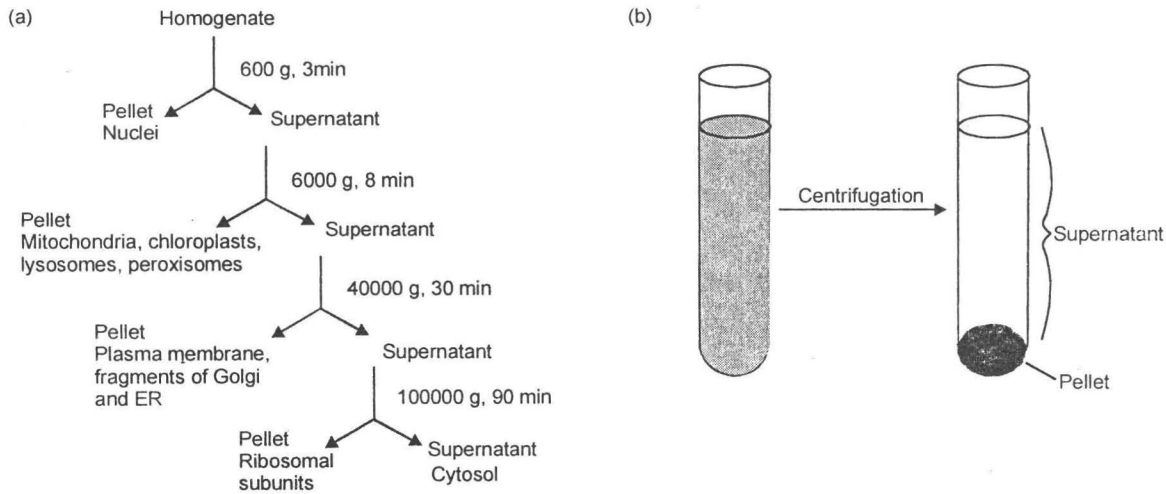

Fig. 5.4. Cell fractionation by differential velocity centrifugation. (*a*) Scheme for subcellular fractionation of a tissue sample, (*b*) appearance of a sample in the centrifuge tube before and after centrifugation.

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2. Equilibrium density-gradient centrifugation : Equilibrium density-gradient centrifugation is generally employed to further purify organelles following their partial separation by differential velocity centrifugation. In this procedure the organelles are separated on the basis of their *density,* not their size. The impure organelle fraction is loaded at the top of a centrifuge tube that is having a gradient of a *dense solution* (*e.g.,* a sucrose solution; Fig. 5.5).

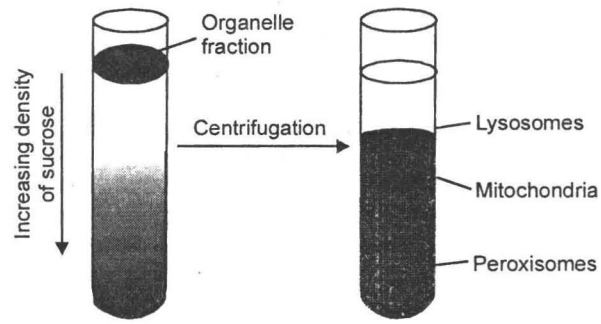

Fig. 5.5. Separation of organelles by equilibrium density-gradient centrifugation.

The sucrose solution has been most concentrated (dense) at the bottom of the tube, and decreases in concentration (and density) towards the top of the tube. During centrifugation (*e.g.,* 160000 *g* for 3 h) the various organelles move down the tube to an *equilibrium position* where their density is equal to that of the sucrose at that position. The *forces of sedimentation* make the organelles move further down the tube but, if they do so, they enter a region of higher density than the organelle density and so they float back to their previous position. Mitochondria, lysosomes and peroxisomes differ in density and so can be efficiently separated from one another by density-gradient centrifugation (Fig. 5.5). Similarly, the rough endoplasmic reticulum, Golgi apparatus and plasma membrane can be separated using a gradient of lower density. The more dense *cesium chloride* finds use to make the density gradient for the separation of denser particles like DNA, RNA and proteins by equilibrium centrifugation.

3. Rate-zonal centrifugation : In rate-zonal centrifugation the sample is layered at the top of a centrifuge tube that is having a sucrose solution of low concentration. In this case, the sucrose is not being used to separate samples by density but simply serves to prevent convection mixing, and is subjected to centrifugation. The organelles move down the tube at a rate determined by the centrifugal force, their mass, the difference between their density and that of the surrounding solution, and the friction between them and the surrounding solution. On completion of centrifugation, different sized organelles are found in different zones of the centrifuge tube. The sample has to be centrifuged for just long enough to separate the organelles of interest; if centrifuged for too long, all of the organelles will end up in the pellet at the bottom of the tube.

4. Marker enzymes : If the cell homogenate is fractionated, the purity of the different organelle preparations needs to be assessed. One way in which this can be done is by assessing *morphology* in the electron microscope. A more readily available alternative though is to measure the activity of (to assay for) a particular *enzyme* which is characteristic of that organelle and is not found elsewhere in the cell. For example, *catalase* is a good marker enzyme for peroxisomes, *succinate dehydrogenase* for mitochondria, *cathepsin C* or *acid phosphatase* for lysosomes, and *alkaline phosphatase* for the plasma membrane. A good indication of the *purity*/degree of contamination of an organelle preparation can be ascertained by measuring the activity of such enzymes in the various isolated fractions.

Flow Cytometry

Different cells could be identified by measuring the light they scatter, or the fluorescence they emit, when they pass a laser beam in a *flow cytometer.* In a *fluorescence-activated cell sorter* of *FACS* (Fig. 5.6), an instrument based on flow cytometry, cells can be identified and

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separated from each other. The cells of interest are first labeled with an **antibody** which is specific for a particular cell-surface molecule. The antibody is coupled to a fluorescent dye, such that when the individual cells pass a laser beam in single file in a narrow stream, the fluorescence of each cell is measured. A vibrating nozzle then forms tiny droplets each having a single cell which are given a positive or negative charge depending on whether the cell they contain is fluorescing. A strong electric field deflects the different charged droplets into separate containers so that each container eventually possesses a homogenous population of cells with respect to the cell-surface molecule tagged with fluorescent antibody. These homogenous populations can then be used for biochemical analysis or grown in culture. The DNA and RNA content of a cell are also measured by flow cytometry.

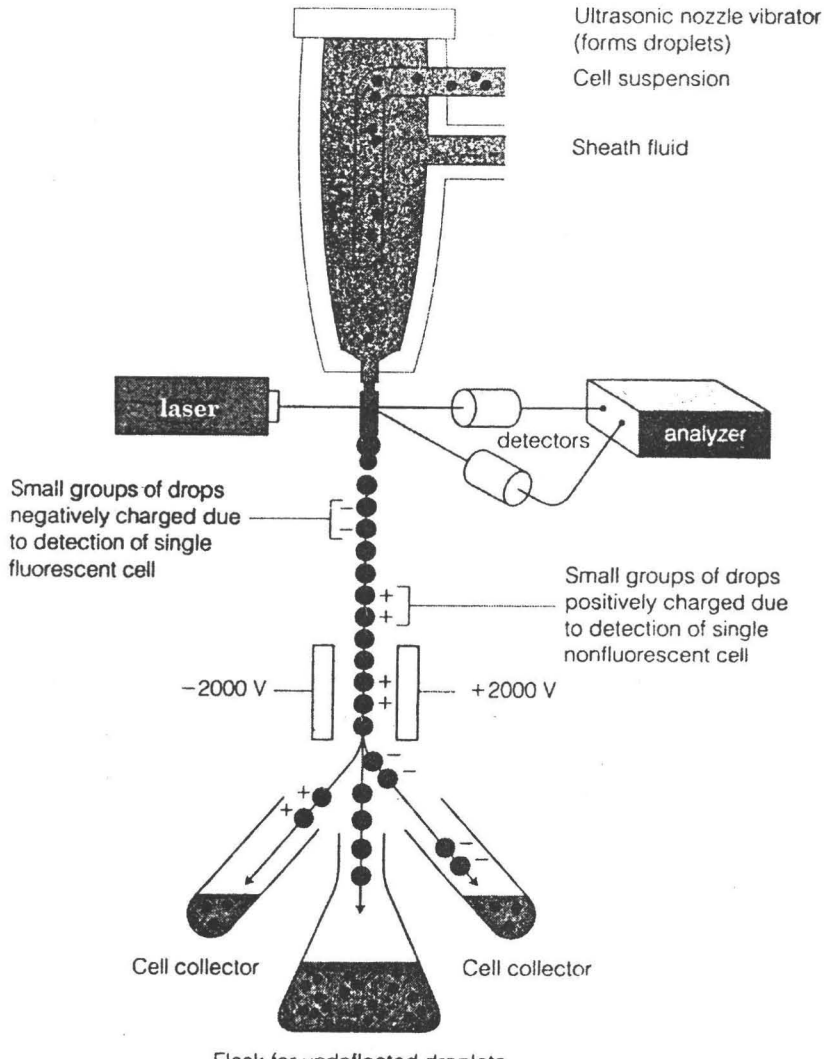

Fig. 5.6. A fluorescence-activated cell sorter. When a cell passes through the laser beam it is monitored for fluorescence. Droplets containing single cells are given a positive or negative charge, depending on whether the cell has bound the fluorescently tagged antibody or not. The droplets containing a single cell are then deflected by an electric field into collection tubes according to their charge.

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5.3 Microscopy

Biochemical analysis is frequently accompanied by light and electron microscopic examination of tissue, cell or organelle preparations to evaluate the integrity of samples and to correlate structure with function. Microscopy serves two independent functions of enlargement (*magnification*) and improved resolution (the rendering of two objects as separate entities? Light microscopes employ optical lenses to sequentially focus the image of objects whereas electron microscopes use electromagnetic lenses. Light and electron microscopes may work either in a *transmission or scanning* mode depending on whether the light or electron beam either pass through the specimen and are diffracted or whether they are deflected by the specimen surface. Polarised light microscopes detect optically active substances in cells, *e.g.*, particles of silica or asbestos in lung tissue, or starch granules in amyloplasts. Light microscopes in *phase contrast* mode are often used to improve image contrast of unstained material, *e.g.*, to test cell or organelle preparations for lysis. Changes in phase of emergent light are caused mainly by either diffraction or by changes in the refractive index of material within the specimen, or even by differences in thickness of the specimen. At their point of focus the converging light rays show interference resulting in either increases or decreases in the amplitude of the resultant wave (*constructive or destructive interference* respectively), which the eye detects as differences in brightness.

Microscopes using visible light will magnify approximately 1500 times and have a resolution limit of about 0.2 p.m. whereas a transmission electron microscope is capable of magnifying approximately 200,000 times and has a resolution limit for biological specimens of about 1 nm. The excellent resolving power of transmission electron microscopy (TEM) is largely a function of the very short wavelength of electrons accelerated under the influence of an applied electric field. (An accelerating voltage of 100 kV produces a wavelength of 4×10^{-3} nm). Scanning electron microscopes (SEM) use a fine beam of electron to scan back and forth across the metalcoated specimen surface. The secondary electrons that are generated from this surface are collected by a scintillation crystal which converts each electron impact into a flash of light. Each light flash inside the crystal is amplified by a photomultiplier and used to build up an image on a fluorescent screen. The principal application of SEM is the study of surfaces such as those of cells. The resolution limit of a scanning electron microscope is about 6 nm.

Optical Microscope

In 1835 Schleiden and Schwann used a primitive light microscope to look at and identify individual cells for the first time. From these studies they proposed their *cell theory* "that the nucleated cell is the unit of structure and function in plants and animals". In light microscopy, *glass lenses* are used to focus a beam of light on to the *specimen* under investigation. The light passing through the specimen is then focused by other lenses to produce a *magnified image*. Technological advances since 1835 have resulted in the manufacture of much more powerful and sophisticated instruments, which have enabled detailed studies of the structure and function of cells to take place.

In the *optical microscope* a *condenser lens* produces a parallel beam of light to illuminate the specimen, and the image is focused and enlarged by *objective* and *eyepiece lenses*. The magnification of each lens is normally stamped on the side so that a $\times$ 10 objective combined with a $\times$ 40 eyepiece gives a total magnification of $\times$ 400. However, the amount of detail which can be seen depends not only upon the magnification but also upon the microscope's ability to distinguish two points close together. If objects are too close, beyond the resolving power, they will blur together no matter what magnification is used. The resolving power (resolution) of a microscope is given by Abbe's formula :

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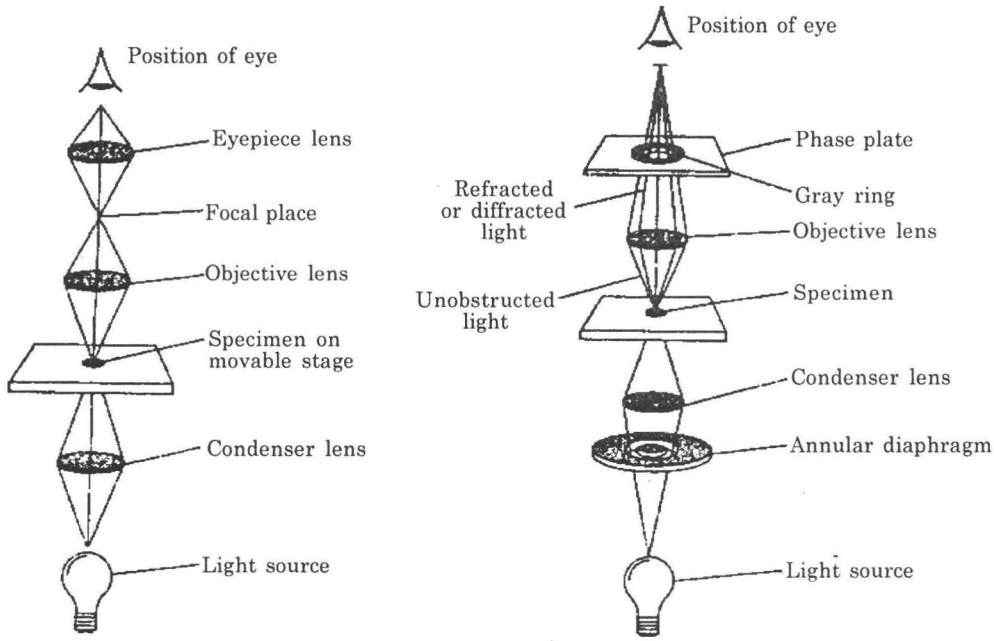

Fig. 5.7. Optical pathway of (*a*) a compound microscope and (*b*) a phase-contrast microscope.

$$R = \frac{0.5\lambda}{n.\sin\alpha}\mu m\,(\text{or}\,nm)$$

where λ = the wavelength of radiation in (or nm).

n = the refractive index (RI) of the medium between the specimen and the first lens.

α = 1/2 angle of the aperture (Fig. 5.7).

(The value $n.\sin\alpha$ is also called the *numerical aperture (NA)* of a microscope. A good quality microscope will have a large NA, *e.g.*, 1.4.)

A small value for R clearly means a better resolution which, according to Abbe's formula, can be obtained by :

(*i*) Making λ smaller, *e.g.*, using short wave (bluish) daylight instead of longer wavelength (Yellowish) artificial light.

(*ii*) Increasing n, by using *immersion oil* (RI = 1.5) to fill the gap between the objective and the specimen, instead of air (RI = 1.0). This requires the use of special *oil immersion lenses.*

(*iii*) Making α as close to 90° as possible, at which point the lens and specimen are in contact.

Most parts of a cell are more or less transparent. At high magnification only a small section of the object is in focus at one time, and a clear image can only be obtained if the specimen is thin. Consequently, the image often appears unnaturally flat. A three dimensional drawing can be constructed, however, by using serial sections. These are thin sections of a structure cut sequentially and viewed in turn.

Standard (bright-field) light microscopy is the most common microscopy technique in use today and uses a *compound microscope.* The specimen to be examined is first fixed with a solution containing alcohol or formaldehyde. These compounds denature proteins and, in the case of formaldehyde, introduce covalent cross-links between amino groups on adjacent molecules which stabilize protein-protein and protein-nucleic acid interactions. The *fixed specimen* is then embedded

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in paraffin wax and cut into *thin sections* (approximately 1 μm thick). Each section is mounted on a glass slide and then positioned on the movable specimen stage of the microscope. The specimen is illuminated from underneath by a lamp in the base of the microscope [Fig. 5.7 (*a*)] with the light being focused on to the plane of the specimen by a *condenser*.

Staining : The various subcellular constituents (nucleus, mitochondria, cytosol, etc.) absorb about the same degree of visible light, making it difficult to distinguish them under the light microscope without first staining the specimen. Many *chemical stains* bind to biological molecules; for example, *eosin* and *methylene blue* bind to proteins, and *fuchsin* binds to DNA. Another useful way of visualizing specific structures within cells is *cytochemical staining* in which an enzyme reaction catalyzes the production of a coloured precipitate from a colourless precursor. The coloured precipitate can then be seen in the light microscope wherever the enzyme is present. For example, peroxisomes can be visualized by using a cytochemical stain for catalase.

Variations in Optical Microscopy

(a) Dark-field microscopy : In dark-field microscopy, light is directed from the condenser lens at an angle so that none of the incident light enters the objective lens; only light *refracted* (bent) or *diffracted* (scattered) by the specimen can enter the lens. The resolution of dark-field microscopy is not particularly good, but this method does allow small objects that refract a large proportion of the incident light to appear as bright particles, and so it is widely used in microbiology to detect bacteria.

(b) Phase-contrast microscopy : In phase-contrast microscopy, a glass *phase plate* between the specimen and the observer further increases the difference in contrast. The incident light is passed through an *annular diaphragm* which focuses a circular ring of light on the specimen [Fig. 5.7 (*b*)]. Light that passes unobstructed through the specimen is focused by the objective lens on to the gray ring in the phase plate which absorbs some of it and alters its phase. Light refracted or diffracted by the specimen will have its phase altered and will pass through the clear region of the phase plate. The refracted and diffracted light waves then recombine with the unrefracted light waves, producing an image in which the degree of brightness or darkness of a region of the specimen depends on the *refractive index* of that region. Phase-contrast microscopy is useful for examining the structure and movement of larger organelles (nucleus, mitochondria, etc.) in living cell but is suitable only for single cells or thin cell layers.

(c) Immunofluorescence microscopy : In immunofluorescence microscopy, the light microscope is adapted to detect the light emitted by a *fluorescent compound,* that is a compound which absorbs light at one wavelength (the *excitation wavelength*) and then emits light at a longer wavelength (the *emission wavelength*). Two commonly used compounds in fluorescent microscopy are *rhodamine*, which emits red light, and *fluorescein*, which emits green light. First, the fluorescent compound is chemically coupled to an *antibody* specific for a particular proteins or other macromolecule in the cell under investigation. Then the fluorescently tagged antibody is added to the tissue section or permeabilized cell, and the specimen is illuminated with light at the exciting wavelength. The structures in the specimen to which the antibody has bound can then be visualized. Fluorescence microscopy can also be applied to living cells, which allows the movement of the cells and structures within them to be followed with time.

(d) Confocal scanning microscopy : Confocal scanning microscopy is a refinement of normal immunofluorescence microscopy which produces clearer images of whole cells or larger specimens. In normal immunofluorescence microscopy, the fluorescent light emitted by the compound comes from molecules above and below the *plane of focus*, blurring the image and making it difficult to determine the actual three-dimensional molecular arrangement. With the confocal scanning microscope, only molecules in the plane of focus fluoresce due to the use of a focused *laser beam*

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at the exciting wavelength. The laser beam is moved to different parts of the specimen, allowing a series of images to be taken at different depths through the sample. The images are then combined by a computer to provide the complete three-dimensional image.

Electron Microscope

Electrons have two basic properties which together made possible the development of *electron microscopes (EMs)* during the 1930s.

(*i*) They are *charged particles* so they respond to *magnetic fields*. In EMs, powerful magnets are used to focus electrons in a similar way to the glass lenses which focus light rays in the optical microscope.

(*ii*) They have *wavelike properties*, this means they can be used to form an *image* on a *fluorescent screen* (the electrons are themselves invisible).

The wavelength of an electron is variable but always minute. It is given by the formula :

$$\lambda = \frac{1.23}{\sqrt{E}} \text{ nm}$$

where E is the voltage through which an electron is accelerated.

Clearly, the higher the voltage the shorter the wavelength, and since Abbe's formula applies equally to both optical and electron microscopes, the best resolution is achieved at high voltages, However, the benefits obtained by using very short wavelengths are partly offset by the aperture in EMs (2α) being much less than 1°, for technical reasons. The net result is that the resolution of the most powerful EMs is about 0.5 nm (0.0005 μm), or 400 × greater than the best optical microscopes. This makes a maximum practical magnification of about 500,000×.

Electrons are easily scattered by air so EMs are usually operated at *high vacuum*. This means that electronmicroscopists are normally restricted to viewing dead, dried specimens. A further difficulty is that at the high voltages needed for the best resolutions, electrons tend to pass straight through the specimen leading to loss of contrast. Contrast can be improved, however, by using heavy metal stains which impede or deflect the electrons and result in the formation of shadows.

There are in fact two main types of electron microscopes :

(a) Transmission electron microscopy : In transmission electron microscopy, a beam of electrons is directed through the specimen and electromagnetic lenses are used to focus the *transmitted electrons* to produce an image either on a viewing screen or on photographic film [Fig. 5.8 (*a*)]. As in standard light microscopy, thin sections of the specimen are viewed. However, for transmission electron microscopy the sections must be much thinner (50-10 nm thick). Since electrons pass uniformly through biological material, unstained specimens give very poor images. Therefore, the specimen must routinely be stained in order to scatter some of the incident electrons which are then not focused by the electromagnetic lenses and so do not form the image. *Heavy metals* such as gold and osmium are often used to stain biological materials. In particular *osmium tetroxide* preferentially stains certain cellular components, such as membranes, which appear black in the image. The transmission electron microscope has sufficiently high resolution that it can be used to obtain information about the shapes of purified proteins, viruses and subcellular organelles.

Antibodies can be tagged with electron-dense gold particles in a similar away to being tagged with a fluorescent compound in immunofluorescence microscopy, and then bound to specific target proteins in the thin sections of the specimen. When viewed in the electron microscope, small dark spots due to the gold particles are seen in the image wherever an antibody molecule has bound to its antigen and so the technique can be used to localize specific antigens.

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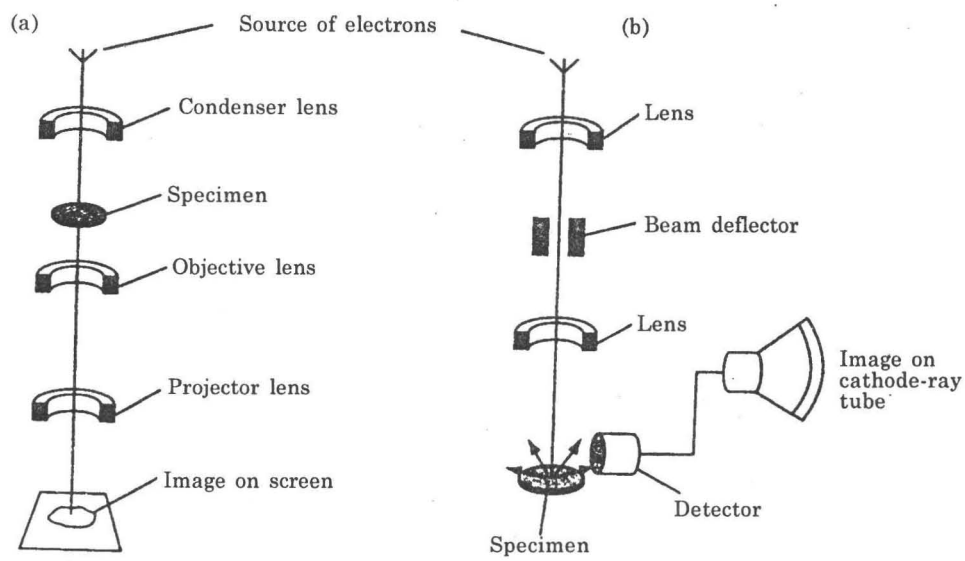

Fig. 5.8. Principal features of (*a*) a transmission electron microscope and (*b*) a scanning electron microscope.

(b) Scanning electron microscopy : In scanning electron microscopy, an (unsectioned) specimen is fixed and then coated with a thin layer of a *heavy metal* such as *platinum*. An electron beam then *scans* over the specimen, exciting molecules within it that release secondary electrons. These secondary electrons are focused on to a scintillation detector and the resulting image displayed on a cathode-ray tube [Fig. 5.8 (*b*)]. The scanning electron microscope produces a *three-dimensional image* because the number of secondary electrons produced by any one point on the specimen depends on the angle of the electron beam in relation to the surface of the specimen. The resolution of the scanning electron microscope is 10 nm, some 100-fold less than that of the transmission electron microscope.

In TEMs electrons are transmitted either through or past the specimens into a fluorescent viewing screen. In SEMs electrons bounce off the heavy-metal shadowed surface of a specimen, and the resulting image is viewed on a television screen. SEMs give by far the best images of surface features with a depth of field over 300× better than optical microscopes. Against this, resolution (10nm) and effective magnification (×20 000) are rather poorer than TEMs.

Preparation of Samples for Electron Microscopy

As Fig. 5.9 suggests, preparing material for electronmicroscopy is a skilled, time-consuming operation. Despite these problems, or perhaps because of them, electronmicroscopists become ecstatic when the shadow of a platinum-plated replica from a dried-up slice of dead cell appears on their viewing screens. Whether what they are looking at bears any resemblance to the structure found in live cells, or is merely an aberration caused by specimen preparation (an *artefact*) had led to heated arguments.

Comparison of Optical and Electron Microscopes

A summary of the main features of both OMs and EMs is given in Table 5.1. As the table suggests, the two forms of microscopy are complementary. Whilst electronmicroscopy reveals fine detail, the use of the optical microscope for rapid viewing of live specimens in colour remains unrivalled.

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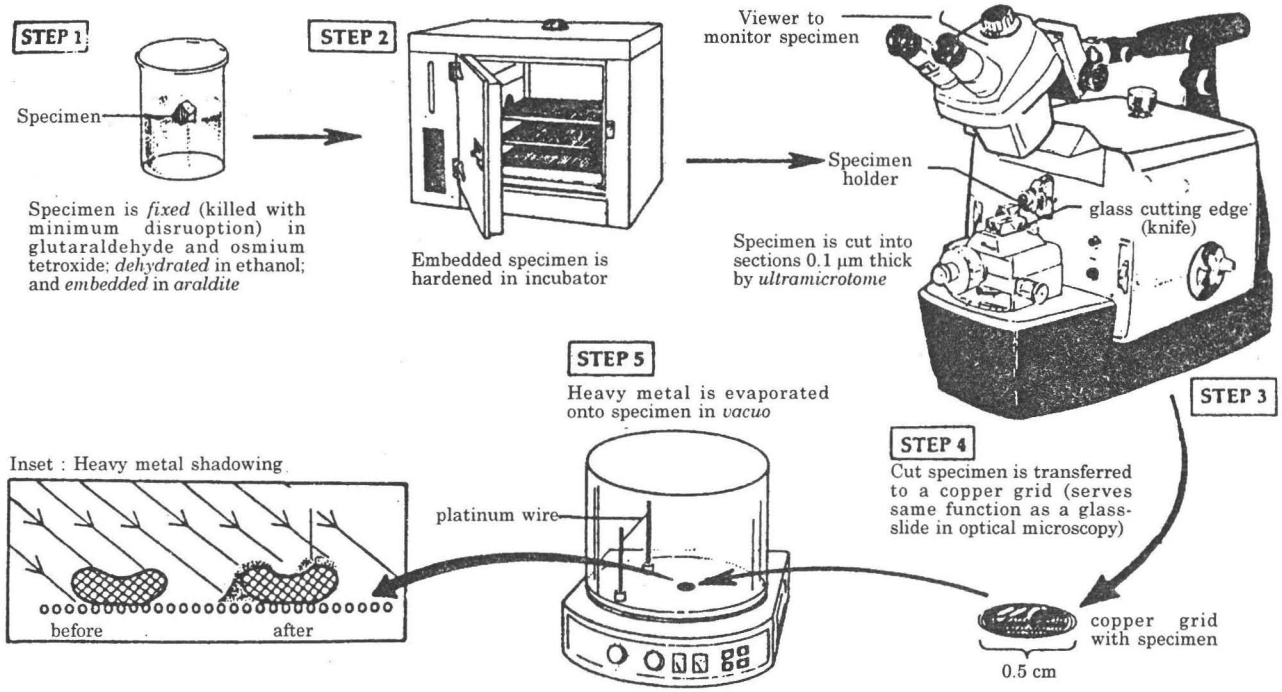

Fig. 5.9. *Preparation of a thin section for use with a transmission electron microscope.* In this example the section is being *shadowed* (inset) by heavy metals. An alternative is to stain the specimen : this would be done after *fixation* (Step 1), and the specimen would then be viewed after transferring it to a copper grid (Step 4).

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Table 5.1
COMPARISON OF OPTICAL AND ELECTRON MICROSCOPY

	Optical microscopy	*Electron microscopy*
1.	Much lower capital outlay and negli gible running costs.	High capital outlay and heavy running costs.
2.	Most techniques can be easily acquired.	High level of technical skill required.
3.	Sample preparation normally takes a few minutes/hours.	Sample preparation often takes several days.
4.	Live or dead specimens may be used. Dehydration not always necessary.	Dead, dried specimens are used.
5.	Colour or black and white.	Black and white only.
6.	Poor at visualising surfaces.	Good surface features (SEMs) or internal detail (TEMs).
7.	Poor depth of focus.	SEM depth of focus 300× better than optical microscopy.
8.	Resolution 170 nm at best. Magnification 2000 at best.	Resolution 0.5nm at best. Magnification × 500000 at best.

Ion Probe Analysis

Electron microscopes may be equiped to perform *X-ray spectrochemical analysis*. When a specimen is irradiated by an electron beam, an electron may be displaced from an inner to outer orbital. When this happens the vacated orbital may be infilled by an electron from a higher energy orbital with the emission of an X-ray photon characteristics of the difference in energy levels on the two orbitals. The binding energy of the electrons in an orbitals is related to the charge on the nucleus; hence each element produces its own characteristic emission spectrum. Emitted photons are usually detected by *energy-dispersive analysis,* using lithium drifted solid-state detectors. Each photon emitted reacts with silicon atoms to produce an electrical pulse proportional to the energy of the photon. An electronic pulse height analyser and microcomputer are used to process the spectral data which is normally displayed on a video monitor. X-ray spectrochemical analysis permits the measurement on ion distributions *in situ* in SEM and TEM by combining the high spatial resolution of electron microscopy with the ability to determine subcellular elemental composition. Areas as small as 100 nm^2 can be analysed under optimal conditions and detection of any element with an atomic number greater than 10 is possible. Measurement is made in terms of total concentration of the element rather than free ion activity and in this sense the method is comparable to flame photometry though with a higher sensitivity.

5.4 Chromatography

Introduction : Chromatography is relatively a new technique which was first invented by *Michael Tswett,* a Russian botanist in 1906 in Warsaw, for the separation of coloured substances into individual components. Since then, the technique has undergone tremendous modifications so that nowadays various types of chromatography are in use to separate almost any given mixture, whether coloured or colourless, into its constituents and to test the purity of these constituents.

The name chromatography (Greek *Chroma* colour and *Graphy* writing) means colour writing.

Chromatography may be defined as the uniform percolation of a fluid through a column of

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more or less uniformly divided substance which selectively retards by whatever means certain components of the fluid (A.F.P. Martin).

In other words, chromatography may be defined as a technique for the separation of the components of mixtures by a continuous distribution of the components between two phases, one of which is moving past the other. The systems associated with this definition are as follows :

(*i*) A solid stationary phase with a liquid or gaseous mobile phase; and

(*ii*) A liquid stationary phase with a liquid or gaseous mobile phase.

The system (*i*) gives rise to adsorption chromatography while the system (*ii*) to partition chromatography.

Theory of Chromatography

Chromatography is essentially based on the distribution of solutes between solvents—a mobile and a stationary. The ratio of the amount of solute retained in one solvent (usually the stationary) to the amount of same solute in the other solvent (mobile) measured under the constant conditions of temperature is termed as the distribution ratio (D). This ratio has been found to depend upon the nature of the solute to be distributed between two solvents. Three types of distributions are obtained which are shown in Fig. 5.10.

The solvent phase in chromatography may belong theoretically to any one of three states of matter (solid, liquid and gases) but the most common employed is the liquid. When Fig. 5.10 is used in terms of distribution, it can be said that each component of a mixture has a definite speed of migration in a definite solvent. As a result of this long chain amino acids migrate more rapidly than glycols and monosaccharides quicker than disaccharides. Thus, a substance may be characterised by the speed at which it gets migrated.

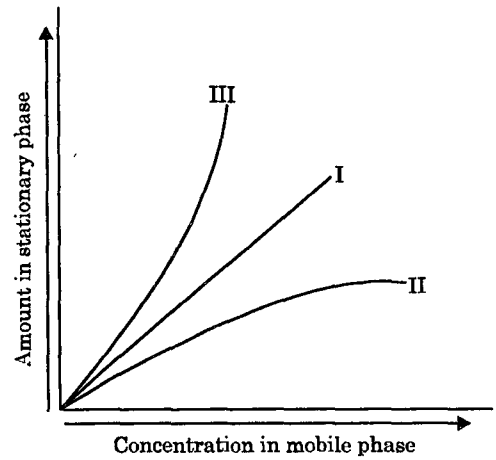

Fig. 5.10. Distribution of solute between two phases of solvents. I—Distribution has been found to be independent of concentration. II—Distribution has been forced to decrease with increasing concentration. III—Distribution has been found to increase with concentration.

Classification of chromatography : The moving phase may be a liquid or a gas. Based on the nature of the fixed and moving phase, different types of chromatography are as follows :

(a) Adsorption chromatography : It is based on the differences in the adsorption coefficients. In this fixed phase is a solid, *e.g.*, alumina, magnesium oxides, silica gel, etc. The solutes are adsorbed in different parts of the adsorbent column. The adsorbed components are then eluted by passing suitable (solvents) through the column.

(b) Partition chromatography : It operates by mechanism analogous to counter-current distribution. The fixed phase may be a liquid strongly adsorbed on a solid which acts as support. In this case, the solute gets distributed between the fixed liquid and the moving liquid (solvent). The technique is called partition chromatography.

Paper chromatography is a special case of partition chromatography in which the adsorbent column is a paper strip.

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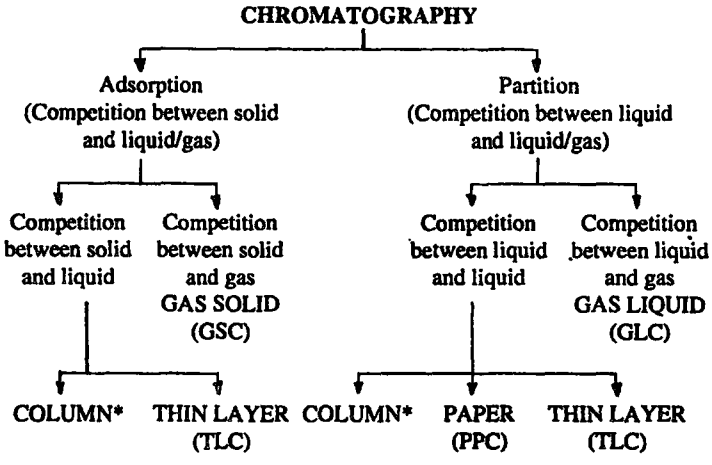
(c) Gas Chromatography : When the moving phase is a mixture of gases, it is called gas chromatography or vapour phase chromatography (VPC).

The various forms of chromatography have been represented schematically as follows :



Various forms of chromatography.

*Other than gas chromatography.

A few chromatographic techniques are described below.

5.5 Paper Chromatography

1. Introduction : The credit for the present full-fledged status of paper chromatography in the realm of separation techniques goes to the Cambridge school of workers, *A.J.P. Martin* and his coworkers *R. Consden, A.H. Gordon* and *R.L.M. Syngle.*

2. Principle : This technique is a type of partition chromatography in which the substances are distributed between two liquids, *i.e.*, one is the stationary liquid (usually water) which is held in the fibres of the paper and called *stationary phase;* the other is the moving liquid or developing solvent and called the moving phase. The components of the mixture to be separated migrate at different rates and appear as spots at different point on the paper.

Originally paper chromatography was used to separate the mixture of organic substances such as dyes and amino acids only. But now this method has been perfected to separate cations and anions of inorganic substances as well.

Procedure : In this technique, a drop of the test solution is applied as a small spot on a filter paper and the spot is dried. The paper is kept in a close chamber and the edge of the filter paper is dipped into a solvent called *developing solvent.* As soon as the filter paper gets the liquid through its capillary axis and when it reaches the spot of the test solution (a mixture of two or more substances), the various substance are moved by solvent system at various speeds. When the solvent has moved these substances to a suitable height (15-18 cm), the paper is dried and the various spots are visualised by suitable reagents called *visualising* reagents. The movement of substances relative to the solvent is expressed in terms of R_F values, *i.e.*, migration parameters.

Migration parameters : The position of migrated spots on the chromatograms are indicated by different terms such as R_F, R_X, R_M and R_C. These parameters are also qualitative and quantitative parameters, characteristic of a substance.

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R_F : The R_F is related to the migration of the solute front relative to solvent front as :

$$R_F = \frac{\text{Distance travelled by the solute from the origin line}}{\text{Distance travelled by the solvent from the origin line}}$$

R_F is function of the partition coefficient. It is a constant for a given substance, provided the conditions of chromatographic system are kept constant with respect to temperature, type of paper, duration and direction of development, nature and the shape and the size of the wick used (*i.e.*, radial chromatography), the amount of liquid in the reservoir humidity, etc.

The R_F defines the movement of the substance relative to the solvent front in a given chromatographic system (Fig. 5.10).

The R_F value of a substance depends upon number of factors which are :

(*i*) The solvent employed,

(*ii*) The medium used for separation, *i.e.*, the quality of paper in case of paper chromatography,

(*iii*) The nature of the mixture,

(*iv*) The temperature, and

(*v*) The size of the vessel in which the operation is carried out.

Keeping the above factors constant, it is possible to compare the R_F values of different substances.

R_X : In some cases, the solvent front runs off the end of filter paper, the movement of a substance in such cases is expressed as R_X but not R_F.

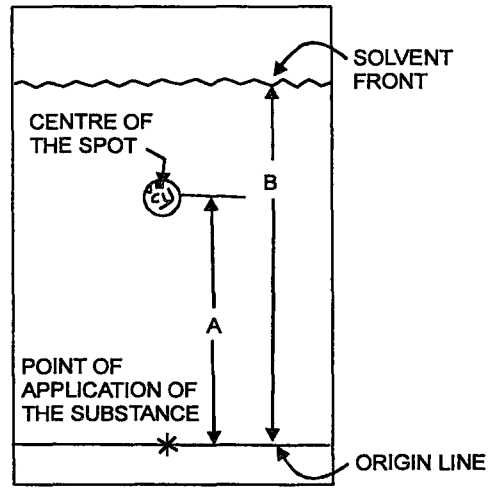

Fig. 5.11. Diagrammatic representation of R_F.

$$R_X = \frac{\text{Distance travelled by the substance from the origin line}}{\text{Distance travelled by the standard substance } x \text{ from the origin line}}$$

Pictorial representations of R_F and R_X have been made in Fig. 5.11 and 5.12.

Types of Paper Chromatography

1. Descending chromatography : When the development of the paper is done by allowing the solvent to travel down the paper, it is known as *descending technique* (Fig. 5.13).

2. Ascending chromatography : When the development of the paper is done by allowing the solvent to travel up the paper, it is known as ascending technique (Fig. 5.14).

Both ascending and descending techniques have been employed for the separation of biochemical substances. But the descending technique is preferred if the R_F values of various constituents are almost same.

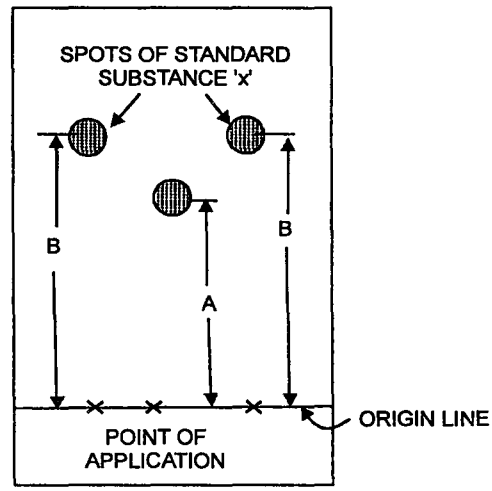

Fig. 5.12. Diagrammatic representation of R_X.

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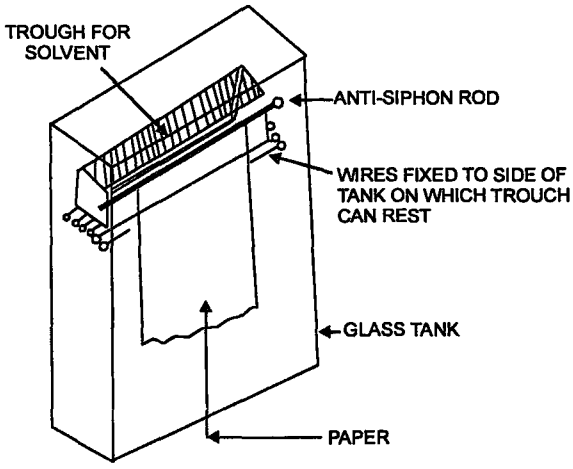

Fig. 5.13. Assembly for descending chromatography.

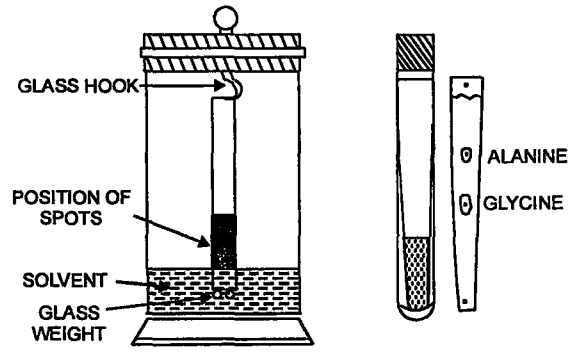

Fig. 5.14. Paper chromatography involving ascending technique.

3. Ascending-descending Chromatography : It is the hybrid of the above two techniques. In this technique, the upper part of the ascending chromatography can be folded over a glass rod allowing the ascending development to change over into the descending after crossing the glass rod (Fig. 5.15).

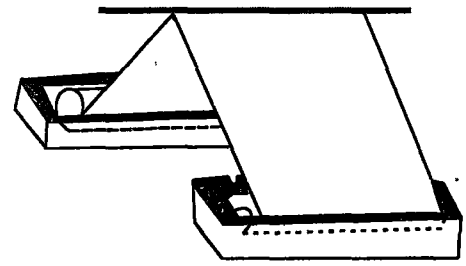

Fig. 5.15. Ascending-descending chromatography.

4. Radial paper chromatography : It is also known as circular paper chromatography. This makes use of radial development. In this technique a circular filter paper is employed. Then the various materials to be analysed are placed at its centre. After drying the spot the paper is fixed horizontally on the petri-dish possessing the solvent so that the tongue or the wick of the paper dips into the solvent. The paper is covered by means of petri-dish cover. The solvent rises through the tongue or the wick. When solvent front has moved through a sufficient large distance, the components get separated in the form of concentric circular zones (Fig. 5.16).

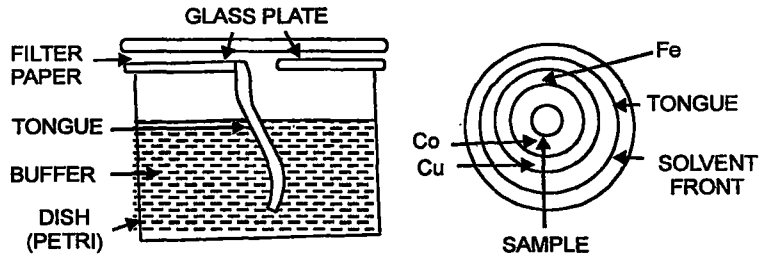

Fig. 5.16. Radial paper chromatography.

5. Two dimensional chromatography : In this, a square or rectangular paper is used. The sample is applied to one of the corners. The second development is performed at right angle to the direction of the first run. This type of chromatography can be carried out with identical solvent systems in both the directions or by two solvent systems. Rack arrangement for the two dimensional chromatography is shown in Fig. 5.17.

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An interesting example of two-dimensional chromatography is the separation of 20 amino acids in the protein hydrolysate.

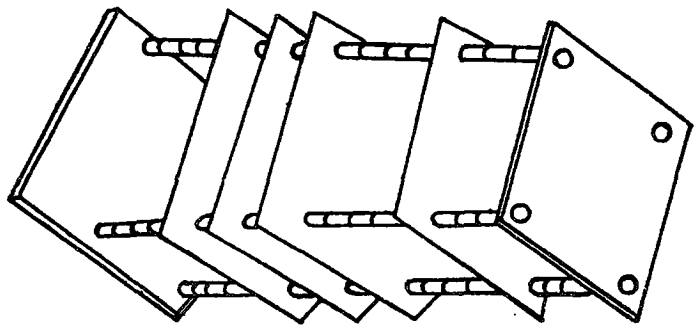

Fig. 5.17. Rack arrangement for two-dimensional chromatography.

Experimental Details for Quantitative Analysis : For quantitative analysis, the preliminary separation is done in the same way as in qualitative analysis. Then, the assay can be performed either after extraction from the paper or *in situ* on the paper.

Estimation After Extraction from the Paper

Isolation of separated components from paper chromatography : The simplest procedure is to cut out the appropriate part of the filter paper having spot and to soak in the minimum quantity of the solvent. Generally, a semi-micio extractor similar to the Soxhelt is being used. The separation of substance from the spot is known as eluation.

Determination : The microanalysis of the resultant eluate can be performed by adopting one or more of the following techniques :

(*i*) Gravimetric estimation, (*ii*) UV spectrophotometry, (*iii*) Colorimetry, (*iv*) Fluorimetry, (*v*) Polarography, (*vi*) Coulometry, (*vii*) Radioactivity, (*viii*) Flame photometry.

In the assessment of the merits of any procedure, the following information is required.

(*i*) The nature of the substance to be assayed,

(*ii*) The scientific equipment available and its sensitivity,

(*iii*) The time available, and

(*iv*) The alternative methods, available, if any, and their relative accuracies.

***In situ* Methods**

(*i*) *By visual assessment* : The simplest procedure is to see the spot by the human eye. However, it is not very quantitative.

(*ii*) *By measurement of areas* : If the outlines of the spots or zones are well defined, the size of the spot (length or areas) may serve for determinating the quantity of the substance. Then a liner relationship is obtained between the spot area and amount of the substance present.

The random errors in this procedure are generally high as a result of the variations of the spot shape during the separation. Other drawbacks of this method are that the volume of applied sample and the speed of application should be identical in all cases.

(*iii*) *By Densitometer* : Densitometer is a method whereby the intensity of the colour of a substance is measured directly on the chromatogram. Various workers have used densitometer in the determination of number of organic compounds.

(*iv*) *Potentiometry* : Changes in the potential of a metal electrode in contact with the filter paper is also utilised with quadrant electrometer of electronic voltmeters.

Besides the above methods, other suitable methods are conductivity, absorptiometry, polarography, etc.

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5.6 Thin Layer Chromatography

Thin layer chromatography is a more recent development than paper chromatography. Its greatest single advantage is versatility in the use of stationary phases.

Thin layer chromatography is adsorption chromatography performed on open layers of adsorbent materials supported on glass plates.

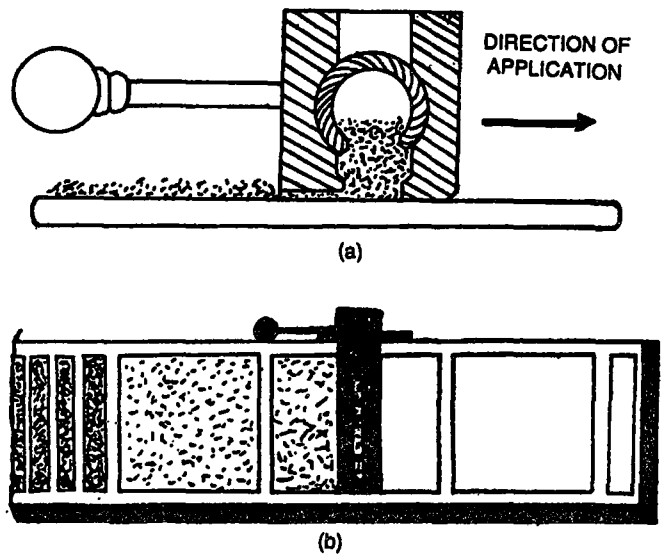

Fig. 5.18. (*a*) Operation of thin-layer spreader. (*b*) Aligning tray with glass slide partially coated (dots = adsorbent).

In this method, a glass plate is coated, by means of a special device (shown in Fig. 5.18), with the material that will constitute the stationary phase mixed with calcium sulphate and made in to a slurry or thin paste sets as a hard surface after drying.

The apparatus developed by Stahl for getting stationary phase consists of two parts : the aligning tray in which the plates are set in a line, and spreader with takes up the spreading mixture and applies it uniformly on a thin layer. The method of operation is shown in Fig. 5.18.

An improved TLC was developed by DESAGA (West Germany). By this new TLC spreader, the layer thickness can be adjusted from 0.0 to 2.0 mm.

The spreading method is the most important nowadays to obtain thin and uniform layers.

The development tank used in paper chromatography is the most common technique in TLC.

In TLC, the plate is placed in a development chamber at an angle of 45° as shown in Fig. 5.19. The bottom of the chamber is covered upto nearly 1 mm by the solvent. Three sides of the tank are lined with solvent impregnated paper while top is covered tightly with the lid as shown.

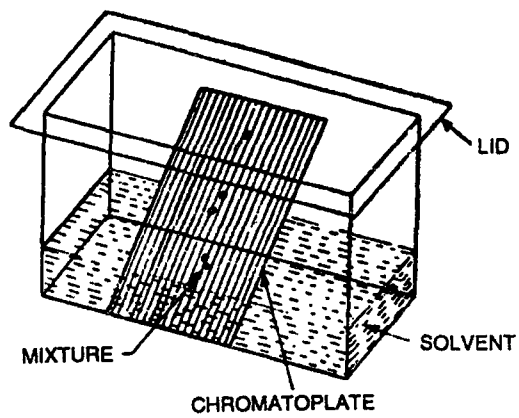

Fig. 5.19. Apparatus for thin layer chromatography.

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The solvent travels upward by capillarity and in a matter of 1 to 2 hours it covers the plate. The plate is removed, dried and otherwise treated just like paper.

Most of the methods used for detecting separated solutes on paper are also applicable to TLC. One can see coloured substances usually. However, one can detect colurless components either under UV light or by treating the plate with a visualising reagent. A large number of visualising reagents are available. The corrosive reagents like chromic acid/sulphuric acid can be used in TLC while these cannot be used in paper chromatography. The possibility of using high temperature techniques such as carbonisation in conjunction with a spray of conc. sulphuric acid offers a universal means of great sensitivity.

In addition to affording a wide choice of stationary phases, thin layer chromatography is fast (1 to 2 hours as against 18 to 24 hours for paper) and has a greater capacity than paper because the layer can be made thicker.

Some interesting applications of thin layer chromatography in biochemistry are given as follows :

(*i*) *Alkaloids* : TLC has been used for the isolation and determination of alkaloids in toxicology where the 30-60 minute runs give a great advantage in comparison to the 12-24 hours required for paper chromatography.

Purine alkaloids have been separated by TLC on silicic acid, silica gel and aluminium oxide. The spots are visualised by spraying first with an alcoholic iodine-potassium iodide solution followed by 25% HCl 96% ethanol (1 : 1).

From lobelia syphilitica, pyridine alkaloids were isolated in a pure form by partition and adsorption chromatography. Partition chromatography was carried out on paper, but adsorption chromatography was carried out on thin layers of silica gel with chloroform-methanol (3 : 1) and on acidic alumina with chloroform-ethanol (19 : 1).

(*ii*) *Amino acids, proteins and Peptides* : *Rokkons* was successful in isolating 34 ninhydin positive substances isolated from urine and separated on silica gel plates. The chromatograms were developed first with chloroform-methanol-17% ammonium hydroxide (2 : 2 : 1) and with phenol-water (3 : 1).

Some cyclic peptides has been separated on thin layers of silica gel using a solvent of benzene-ether-methanol (85 : 10 : 5).

(*iii*) *Antibiotics* : Tetracyclines has been separated on thin layers of silica gel G by using solvents like 10% citric acid *n*-butanol-methanol-10% citric acid solution (4 : 2 : 2) and (4 : 3 : 2).

Penicillins have been separated on silica gel 'G' by using the two solvents, acetone-methanol (1 : 1) and iso-propanol-methanol (3 : 7). As the detecting agent, the iodine-azide reaction was employed by spraying the dried plates with a 0. I N iodine solution containing 3.5% of sodium azide.

Neomycin sulphates have been separated on layers of activated carbon. Both acidic and neutral layers were used. Active carbon is also used for the separation of water soluble basic antibiotics produced by streptomyces. Erythromycins have been separated on thin layers of silica gel 'G' using a solvent composed of methyl chloride-methanol-benzene-formamide (80 : 20 : 20 : 2.5).

From the above example, it can be concluded that the speed, efficiency and sensitivity of thin layer chromatography have made this technique one of the most powerful available to biochemist.

5.7 Ion Exchange Chromatography

Introduction : Ion exchange may be defined as a reversible reaction in which free mobile ions of a solid called ion exchange are exchanged for different ions of similar charge present in solution.

The principle of ion-exchange chromatography is based upon the simple fact that different cations (or anions) have different capacity to undergo exchange reaction on the surface of a given

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exchange. The capacity of an ion to undergo exchange reaction has been found to depend upon the exchange and the size of the hydrated ion in solution. Under similar conditions the capacity has been found to increase with the charge on the ion (*i.e.*, the valency of the ion) but has been found to decrease with the increase in the size of the hydrated ion.

Most ion exchangers for practical use consist of an insoluble organic polymer into which a charged group has been introduced in some suitable manner. The backbone is generally a styrene-divinyl-benzene copolymer. Copolymers of acrylic acid derivatives divinyl-benzene are also frequently used. For use with biological macromolecules, it is usual to introduce charged groups into cellulose fibres. For several years, cross-linked dextran (Sephadex) has been also used as carrier material. Cellulose derivatives such as carboxymethyl cellulose (CMC) and diethylaminoethyl cellulose (DEAE) exchangers have been very successfully used in protein purification.

Apparatus : The apparatus used for ion exchange chromatography is shown in Fig. 5.20. In this figure, a column is provided with a stirrer to mix the ion exchanger and mixture of the substances to be analysed.

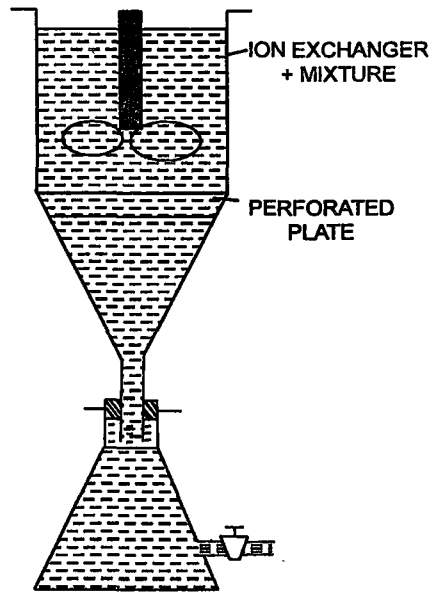


Fig. 5.20. Arrangement for ion exchange chromatography.

In the ion exchange chromatography, the column is filled with any ion exchanger which may be either acidic or basic. When a dilute solution of cation is passed through a column packed with an acid resin, the acid group of resin gets exchanged with this cation. This cation forms complex with resin and it can be recovered by employing an eluting solvent of desired ion strength and pH.

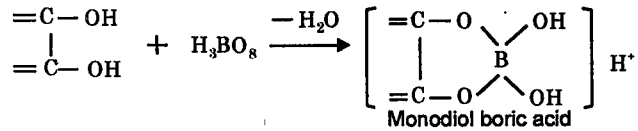
$$\underset{\text{Resin}}{RH^+} + \underset{\substack{\text{Cation}\\ \text{solution}}}{MX^+} \longrightarrow RX + H^+ + M^+$$

The hydrogen ion released may be washed away with the distilled water.

Similarly anion exchange (basic) resins can be employed by using alkalies.

When a mixture of substances is put in the column filled with ion exchanger, then by adjusting the pH of the eluting solvent and the ionic strength, the electrostatically held ions are eluted differentially to yield to desired separation.

Applications : The technique of ion exchange chromatography has been widely used for many biochemical mixtures such as proteins, nucleic acids, vitamins, sugars, amino acids, etc. Some of these are described as follows :



Monodiol boric acid

(i) Separation of sugars : This method was developed by Khym and Zill (1951). The sugars are first of all converted into borate complexes.

Separations of borate complexes of sugars have been achieved on 11 × 0.9 cm columns of 200 – 400 mesh Dowex 1 resin, using a loading of 5 – 10 mg of borate complex and flow rates of 0.5 – 1 ml/min. Quantitative recovery of sugars is possible after separation of the borate complexes.

Similarly, disaccharides can be separated from the monsaccharides and the individual compounds of hexose and pentose mixtures resolved.

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(ii) Separation of amino acids : Ion exchange chromatography has been used to separate the complex mixture of 18 amino acids obtained by the acid hydrolysis of proteins. Fig. 5.21 shows the analysis of a test mixture having all amino acids in molar ratios. The area under the peaks is a measure of the amount of material used which can be employed for calibration purposes.

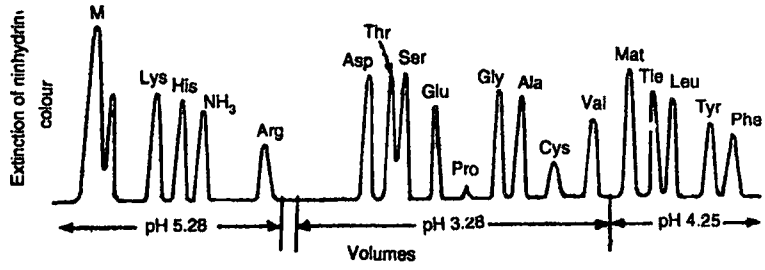

Fig. 5.21. Mixed amino acid in an amino acids analyser chromatographed on special polystryene sulphonic acid resion.

The mixture of amino acids is first introduced into very short column at pH 2 and eluted with 0.35 N sodium citrate buffer at pH 5.25, with which the column has been equilibrated. Acidic and neutral amino acids at first leave the column unseparated as is shown by the first peaks (M). They are followed by lysine, histidine, NH_3 and arginine. Now a second sample is chromatographed into a longer column with 0.2 N sodium citrate buffer at pH 3.28 and subsequently eluted with 0.88 N citrate buffer at pH 4.25.

(iii) Purification of cytochrome c : Cytochrome c has an isoelectric point of pH 10.05. A column containing a cation exchanger buffered at pH 8.5 is prepared. An impure solution of cytochrome at pH 8.5 is placed on the column and water is passed through the column. The contaminating proteins pass freely through the column. (The pI of proteins is usually 7.0 or less) but cytochrome *c* is held firmly by electrostatic attraction to the resin beads. If the eluting solvent pH is now raised to about 10, the cytochrome c will have a net zero charge and will pass rapidly through as a pure component.

(*iv*) Separation of proteins : Proteins are polyionic molecules and like amino acids, most proteins bear a positive charge at acid pH or a negative charge at basic pH. They exchange with one or the other type of cellulose derivative, depending on the pH of the solution. They are selectively eluted from the column by buffers of increasing or decreasing pH. For example, if a mixture of proteins is placed on a CMC (carboxymethyl cellulose) at pH 4, the proteins are eluted from the column by increasing the pH of the buffer. Selective elution can also be accomplished by using solutions of salts of increasing ionic strength. The essential process is depicted in Fig. 5.22.

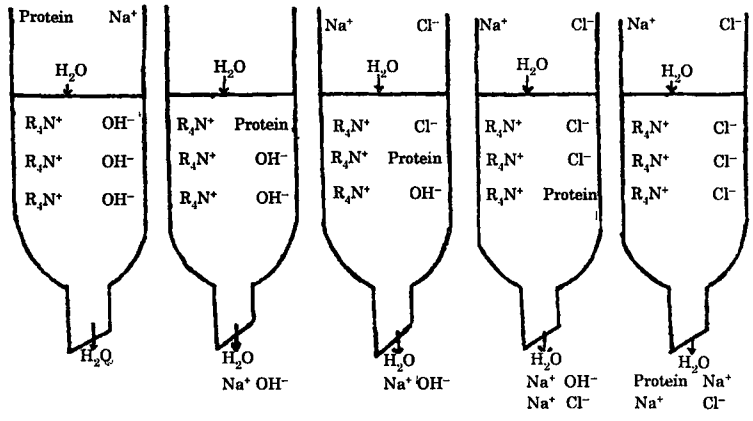

Fig. 5.22. Ion exchange chromatographic separation of proteins.

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5.8 Column Chromatography

Principle : It is known that the rate of adsorption varies with a given adsorbent for different materials. This principle of selective adsorption is used in column chromatography.

In this method, the mixture to be separated is dissolved in a suitable solvent and allowed to pass through a tube containing the adsorbent. The component which has greater adsorbing power is adsorbed in the upper part of the column. The next component is adsorbed in the lower portion of the column which has lesser adsorbing power than the first component. This process is continued. As a result the materials are partially separated and adsorbed in the various parts of the column. The initial separation of the various components can be improved by passing either the original or some other suitable solvent slowly through the column. The various bands present in the column become more defined. The banded column of adsorbent is termed as a *chromatogram*, and the operation is spoken of as the *development of chromatogram*. The portion of a column which is occupied by a particular substance is called its zone. The narrower the zones, the larger the number of substances which can be separated in column of a definite length, and the more concentrated are the eluates.

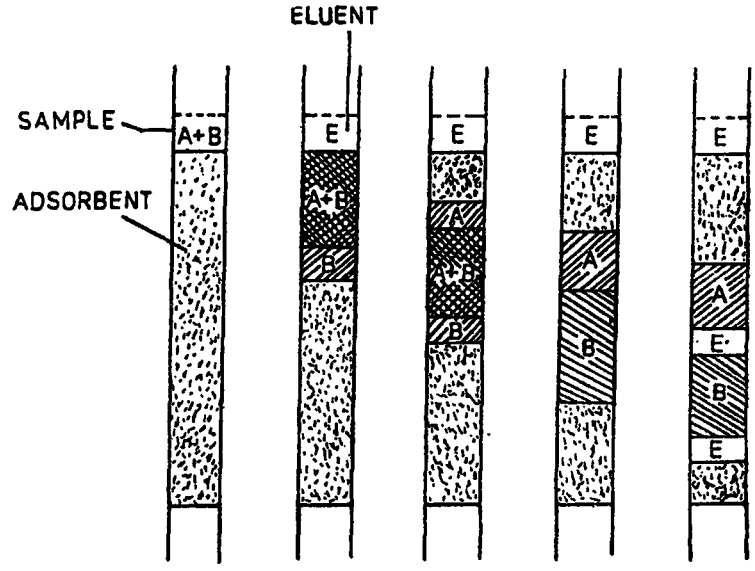

Fig. 5.23. Elution development of two solutes A and B. Hence A is more strongly adsorbed than B. E is the eluent.

In order to separate or to estimate the various constituents, two procedures may be adopted :

(*i*) After development, the column of adsorbent may be pushed out of the tube, the various zones are cut with a knife at boundaries and the substances present in zones extracted with a suitable solvent. This process of recovery of constituents from the chromatogram is known as elution.

(*ii*) After development the column may be washed with more solvent, now termed the eluent, and each component is collected separately as it reaches the end of the column and is released.

The process of separation of a mixture of two substances A and B has been represented in Fig. 5.23.

General requirements : A complete set up for column chromatography is shown in Fig. 5.24.

The essential requirements for adsorption chromatography are a column, an adsorbent and the solvents :

(*i*) The column is generally a glass tube having means for supporting the packed adsorbent and opening for admission and collection of the mobile phase of the solvent. Different types of columns used are depicted in Fig. 5.25.

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(*ii*) The adsorbent should be inert and insoluble in the solvent used. Sometimes, some inert substances like diatomaceous earth, are mixed with relatively active adsorbent for removing the flow characteristics. The adsorbents such as alumina. $CaCO_3$ (for plant pigments), calcium phosphate (proteins), celite or super cell (proteins), cellulose (organic compounds), glass (high m.w. organic compounds), Kaolin (porphyrins, carbohydrate, vitamins A, D and E), magnesium oxide, magnesium silicate (steroids, carbohydrate derivatives), silicic acid (hydrocarbons, amino acids, carbohydrate derivatives) and sucrose (chlorophyll) are commonly used.

(*iii*) The solvents should be pure and stable. Their recovery from the dissolved substances should be easy. Acetone or organic compounds of this type are generally used in chromatographic separation of plant pigments.

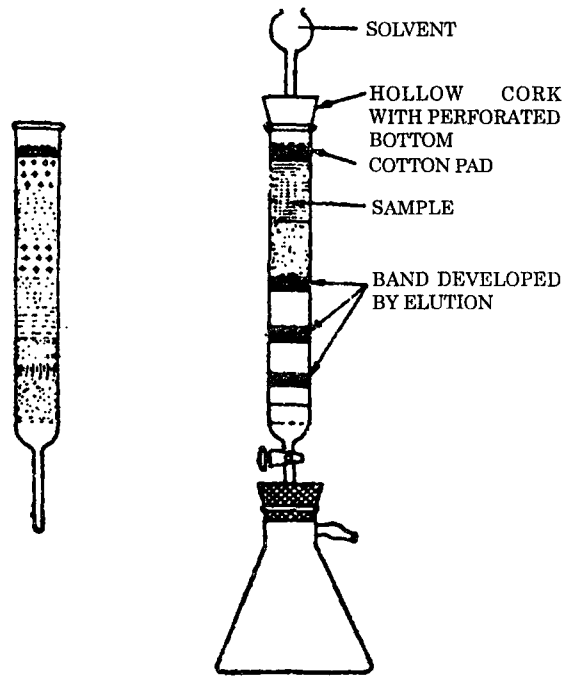

Fig. 5.24. A complete set up for column chromatography

5.9 Gas Chromatography

Introduction : Gas chromatography is quite similar to column chromatography, except that a gas is used as the mobile phase instead of a liquid. Gas-solid chromatography (GCS) is based upon selective adsorption on a solid, whereas gas-liquid chromatography is based upon the partition between the gas and an immobile liquid phase.

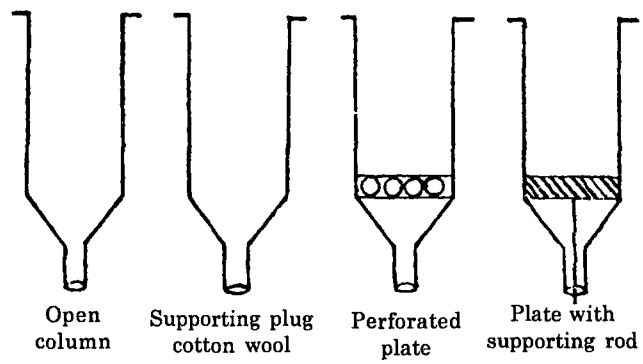

Fig. 5.25. Types of chromatographic columns.

Instrumentation : The basic components of gas chromatographs are shown in Fig. 5.26. It should be noted that these components are same for GSC and GLC. The gas chromatographic separation is carried out in a tubular column made of glass, metal or teflon. In this column adsorbent is filled as the stationary phase. The adsorbents are packed in the form of fine size graded powder, whereas the liquids are either coated as fine film on the column wall or first coated over an inert size graded porous support such as firebrick powder followed by packing in the column. A gas serving as mobile phase, flows continuously through the column. It is known as the carrier gas and serves to transport sample components in the column. The sample is introduced in the vapour form at the carrier gas entrance end of the column. Different components of the sample are sorbed on the stationary phase to different extents depending upon their distribution coefficients. The

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portion of each component in the gas phase is swept further immediately by the carrier gas. As a result a fraction of the sorbed amount also desorbs out to maintain the K-value. At the same time, out of swept amount some amount will go into sorbent at the next point in the column again to maintain the K-value. This goes on successively and continuously and as a whole the band for each component moves further in the column and having the shape of more or less, Guassian distribution. The separation of a binary mixture is shown in Fig. 5.27.

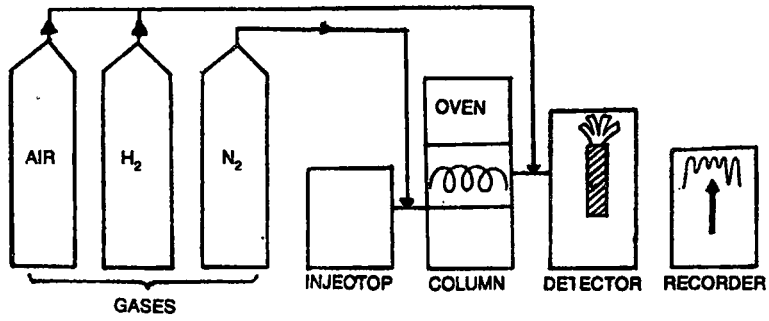
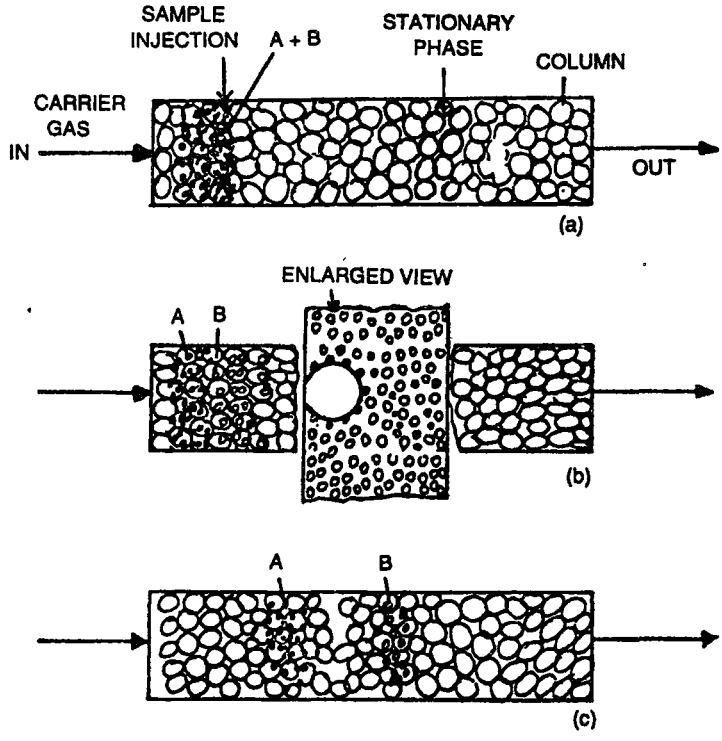


Fig. 5.26. Line diagram of a gas chromatograph.



(*a*) Sample introduction
(*b*) Partial separation achieved
(*c*) Complete separation achieved

Fig. 5.27. Separation of a Binary Mixture (A + B).

It should be kept in mind that the gas is the driving force for the movement of zones through the column and the solid (for GSC or liquids for GLC) provides the selective retarding force. The detector can be regarded as the brain of the chromatograph and the column as its heart.

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Gas chromatography is an extremely sensitive method capable of detecting traces of substances that can be detected by any other means. The reason for its sensitivity lies mainly in the detection system at the outlet of the column. There are several type of detectors that measure some property related to the mass of the substances that emerges from the column.

Applications : Gas chromatography has become one of the most widely used chromatographic methods. It is fully automated and extremely versatile but its uses in biochemistry are limited by the instability of many molecules to the high temperatures employed in gas chromatography. It is successfully applied when the mixtures consist of volatile substances such as fatty acids.

5.10 High Performance (Pressure) Liquid Chromatography

The resolving power of a chromatographic column increases with column length and the number of theoretical plates per unit length, although there are limits to the length of a column due to the problem of peak broadening. As the number of theoretical plates is related to the surface area of the stationary phase it follows that the smaller the particle size of the stationary phase, the better the resolution. Unfortunately, the smaller the particle size, the greater the resistance to eluant flow. All of the forms of column chromatography so far discussed rely on gravity or low pressure pumping systems for the supply of eluant to the column. The consequence of this is that the flow rates achieved are relatively low and this gives greater time for band broadening by simple diffusion phenomena. The use of faster flow rates is not possible because it creates a back-pressure which is sufficient to damage the matrix structure of the stationary phase, thereby actually reducing eluant flow and impairing resolution. In the past decade there has been a dramatic development in column chromatography technology which has resulted in the availability of new smaller particle size stationary phases which can withstand these pressures and of pumping systems which can give reliable flows rates. These developments, which have occurred in adsorption, partition, ion-exchange, exclusion and affinity chromatography, have resulted in faster and better resolution and explain why HPLC has emerged as the most popular powerful versatile form of chromatography.

Originally, HPLC was referred to as high pressure liquid chromatography but nowadays the term high performance liquid chromatography is preferred since it better describes the characteristics of the chromatography and avoids creating the impression that high pressures are an inevitable pre-requisite for high performance. This is now known not to be the case and the term *medium pressure liquid chromatography* (MPLC) has been coined for some separations. The principal components of a HPLC system are shown in Fig. 5.28.

The new technology of stationary phases has been applied to thin-layer chromatography giving rise to high *performance thin-layer chromatography* (HPTLC). In general, however, the impact of this new technology has not been quite so great as it has been in column chromatography.

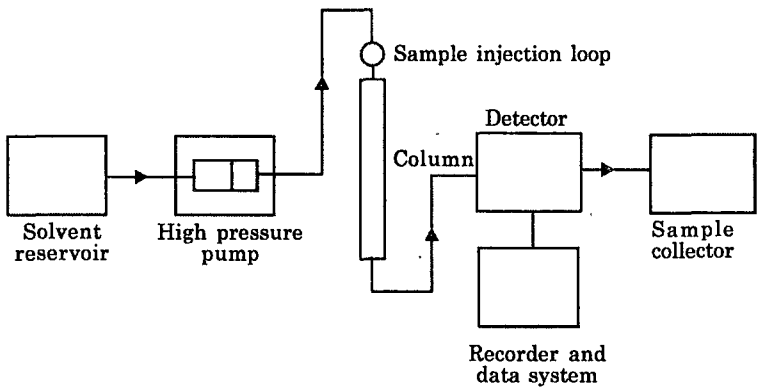

Fig. 5.28. Diagrammatic representation of the components of an isocratic HPLC system.

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Applications

The wide applicability, speed and sensitivity of HPLC has resulted in it becoming the most popular form of chromatography and virtually all types of biological molecules have been purified using the approach. *Reverse phase partition* HPLC is particularly useful for the separation of polar compounds such as drugs and their metabolites, peptides, vitamins, polyphenols and steroids. Prior to the advent of this form of chromatography, the separation of such polar compounds was not easily accomplished and often required pre-derivatisation to less polar compounds. The technique is particularly widely used in clinical and pharmaceutical works as it is possible to apply biological fluids such as serum and urine directly to the column, preferably using a guard column. The separation of some highly polar compounds, such as amino acids, organic acids and the catecholamines which are difficult to resolve, adequately, by reverse phase chromatography, can often be improved by one of two possible approaches. The first is *ion-suppression* in which the ionisation of the compound is suppressed by chromatographing at an appropriately high or low pH. Weak acids, for example, can be chromatographed using an acidified mobile phase. The second is *ion-pairing* in which a *counter* ion with opposite charge to that to be separated is added to the mobile phase so that the resulting ion-pair as sufficient lipophilic character to be retained by the non-polar stationary phase of a reverse phase system. Thus to aid the separation of acidic compounds which would be present as their conjugate anions, a quaternary alkylamine ion such as tetrabutylammonium would be used as the counter ion whereas for the separation of bases which would be present as a cation, an alkyl sulphonate such as sodium heptanesulphonate would be used. The mechanism by which ion-pairing results in better separation is not clear but two theories have been proposed. The first suggests that the ion-pair behaves as a single neutral species, whilst the second suggests that an active ion-exchange surface is produced in which the counter ion, which has considerable lipophilic properties, and the ions to be separated are adsorbed by the hydrophobic, non-polar stationary phase. In practice, the success of the ion-pairing approach is variable and somewhat empirical. The size of the counter ion, its concentration and the pH of the solution are all factors which may profoundly influence the outcome of the separation.

HPLC has probably had the biggest impact on the separation of oligopeptides and proteins. Instruments dedicated to the separation of proteins have given rise to the technique of *fast protein liquid chromatography* (FPLC). There are no unique principles associated with FPLC, it is simply based on reverse phase and ion-exchange chromatography and on chromatofocussing. Microbore glass-lined stainless steel columns 1 mm diameter and 2.5 cm long have recently been developed which enable very small amounts of sample to be used with separation taking as little as 10 minutes. The technique enables such complex mixtures as tryptic digests of proteins and the culture supernatant of microorganisms to be applied directly to the column which most commonly contains an ion-exchange system. Protein mixtures from cell extracts still need some form of preliminary fractionation prior to study. Although high performance exclusion and ion-exchange chromatography are so successful for protein separation, not all proteins can be completely purified using them. In these cases, the technique of *hydrophobic interaction chromatography* which exploits hydrophobic regions on the surfaces of proteins, may be successful. The stationary phase is strongly hydrophobic and most commonly is octyl- or phenylagarose. The hydrophobic regions of the proteins surface interacts with this phase by $\pi - \pi$ interaction. This minimises interaction of the protein with the aqueous environment. Binding is accomplished in dilute (0.0 1 M) buffer and elution carried out either with aqueous ethylene glycol or ethanol or by the addition of so-called *chaotropic compounds* (perchlorate, trifluoroacetate or thiocyanate ions or urea) which disrupt water structure and thus discourage hydrophobic interactions. Proteins purified by this technique include aldolase, transferrin, cytochrame *c* and thyroglobulin.

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5.11 Electrophoresis

The powerful method, which was developed by Tiselius in 1937 is based on the principle that there occurs the movement of charged particles under the influence of an electric current to oppositely charged electrodes.

Many important biological molecules such as amino acids, peptides, proteins, nucleotides and nucleic acids possess ionisable groups and can therefore be made to exist in solution as electrically charged species, either as cations (+) or anions (–). Even typically non-polar substances such as carbohydrates can be given weak charges by derivatisation, for example, as borates or phosphates. Moreover, molecules which have a similar charge will have different charge/mass ratios when they have inherent differences in molecular weight. In combination these differences form sufficient basis for a differential migration when the ions in solution are subjected to an electric field. This is the principle of *electrophoresis.*

The equipment required for electrophoresis consists basically of two items, a power pack and an *electrophoresis unit.* Cations move to the cathode (–) and anions move to the anode (+) at rates which depend on the balance between the impelling force of the electric field on the charged ion and the frictional and electrostatic retarding effects between the sample and the surrounding medium. The sample must be dissolved or suspended in buffer for electrophoresis to take place and any supporting medium must also be saturated with buffer to conduct the current. A buffer is also important to maintain a constant state of ionisation since changes in pH would alter the charge on molecules being separated, particularly when sample ions are zwitterions.

The current is maintained throughout the circuit by electrolysis taking place at the electrodes, both of which dip into large *buffer reservoirs.*

$$2e^- + 2H_2O \xrightarrow{\text{Cathode}} 2OH^- + H_2\uparrow \quad H_2O \xrightarrow{\text{Anode}} 2H^+ + \tfrac{1}{2}O_2\uparrow + 2e^-$$

The movement of charged particles under the influence of an electric field has been found to depend upon time, electric current, conductivity of the solvent and size and charge of the particle. The distance travelled by the particles in one second under a potential gradient of one volt per centimeter is termed as *electrophoretic mobility.* As particles of different sizes are having different electrophoretic mobility, under the influence of some current, it becomes possible to separate them from a mixture. It is also possible to observe electrophoretic movement under the influence of pH gradients also. Amino acids, for example, do not move to any pole at their isoelectric point because they carry a positive charge at lower pH and move towards cathode and at pH values higher than the isoelectric point they carry a negative charge and move towards anode. The acidic, basic and neutral amino acids will move towards poles at different pH values. It becomes therefore difficult, to maintain a constant pH gradient in the presence of electric current.

Experimental : Initially this technique of electrophoresis was used for the separation of proteins in a solution. The separation by this method, was not quite satisfactory, and several types of supporting materials were used. The charged particles move along this medium, which is generally porous. Most common media are filter paper, cellulose acetate, starch gel, agar and polyacrylamide gel. The filter paper is generally used in routine electrophoretic analyses, although it allows mixing of zones to some extent, due to absorption of molecules on the paper.

Commercially available apparatus for electrophoresis are available in two types, the vertical type, in which the supporting medium has been suspended by a glass rod with its end hanging down and the horizontal type in which the medium has been kept horizontal. Essential components of a vertical paper electrophoresis are shown in Fig. 5.29. The apparatus consists of two electrode tanks which are made to connect through the supporting medium (paper in this case). The paper

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strip itself has been supported between two glass rods. The electrodes have been connected with a suitable D.C. source. The electrode tanks are filled with a buffer solution. The solution should be selected carefully as some components of the buffer may react with the compounds under separation. The pH of the buffer depends upon the mixture to be separated. Generally, maximum separation has been attained at the isoelectric point of one of the compounds.

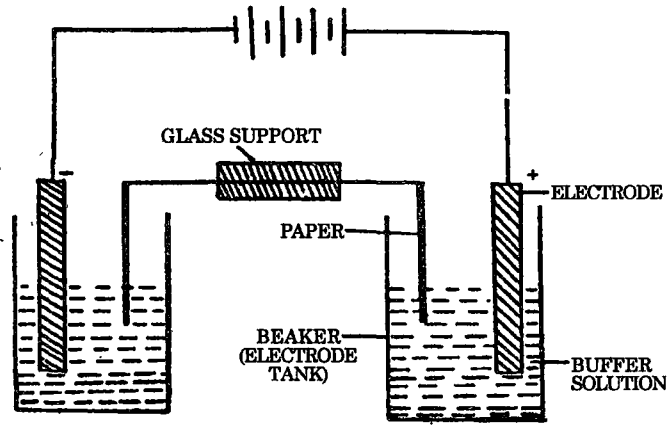

Fig. 5.29. Apparatus and arrangement for paper electrophoresis.

Electrophoresis is mainly used to resolve different components of a protein mixture. Isozymes are also separated by electrophoresis on starch gel or cellulose acetate.

An important modification of the electrophoretic technique described above involves the separation of proteins and the other charged molecules in an electric field passing through a solution supported by inert materials such as moistened filter paper, starch gel, silica gel, cellulose sponges or glass powder. This method is known as zone electrophoresis or ionophoresis and is readily used for separation of different mobility into zones. After separation, it is possible to locate different zones by staining with dyes. Further it is possible to extract the individual components from these zones. Thus, zone electrophoresis is a combination of electrophoresis and chromatography. The zone electrophoresis apparatus using silica gel is depicted in Fig. 5.30.

Zone electrophoresis has been found to be useful in the study of serum proteins and the cleavage products of proteins and nucleic acids.

5.12 High Voltage Electrophoresis (HVE)

When low molecular weight compounds are separated by low voltage paper electrophoresis, considerable diffusion occurs. This can largely be overcome by using much higher voltages, resulting in better resolution and very rapid separations (10 to 60 min). High voltage power packs are available which will supply up to 10000 V and 500 mA, producing potential gradients of up to 200 V cm^{-1}.

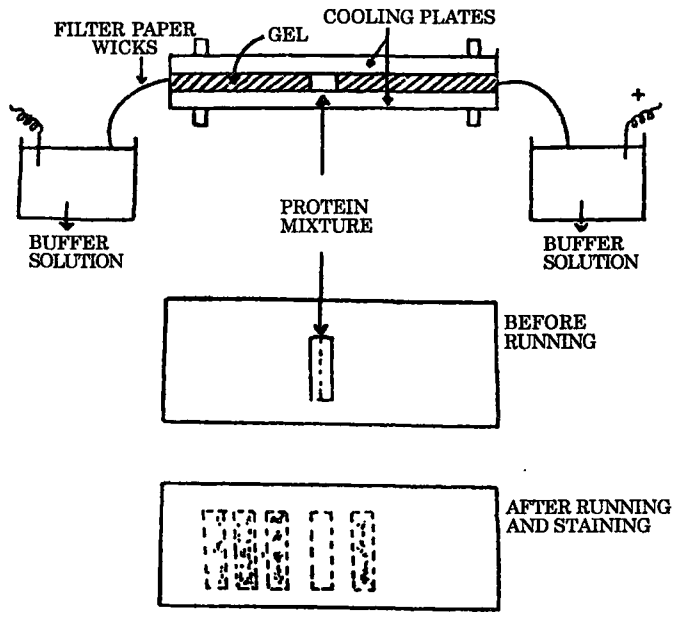

Fig. 5.30. Schematic representation of gel electrophoresis apparatus. (The two lower figures represent separation of protein mixtures).

High voltage electrophoresis generates so much heat that a direct cooling system is required. This is

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normally achieved by using cooling plates (Fig. 5.31). The two plates, normally aluminum, are insulated from the supporting medium by polythene and the plates are pressed against the insulated supporting medium by an inflatable pressure pad. Equipment is available with plates of up to 50 × 50 cm, to enable large sheets of paper (generally Whatman 3 MM) to be used. Cold water is circulated through channels in the cooling plates, and for large plates, a flow rate of between 10 and 15 $dm^3\ min^{-1}$ is required to disperse the heat produced. In high ambient temperature conditions the cooling water should first be refrigerated; in areas where water is very hard, the use of a water softener is recommended in order to avoid furring up the channels in the plates. Temperature gradients across the plates must be avoided, since a difference of 1°C causes a 3% change in migrational velocity, and this affects reproducibility. Complete electrical insulation of the equipment is vital because lethal voltages are used. Normal safety precautions should be strictly observed even though commercial systems have a number of cut-out (fail safe) devices to avoid accidents.

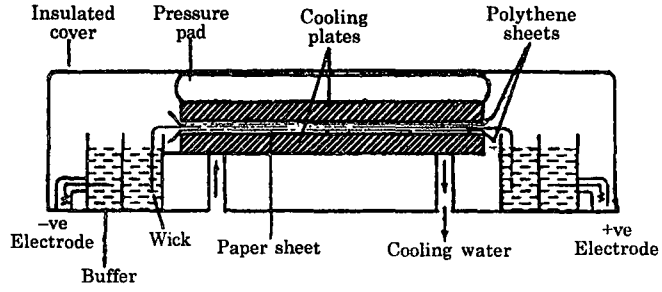

Fig. 5.31. High voltage electrophoresis (HVE) unit.

For the separation of protein hydrolysates, HVE in one dimension followed by chromatography in a second dimension is frequently used. This two-dimensional system allows characteristic fingerprints of different proteins to be obtained, as with two-dimensional thin layer electrophoresis. HVE has proved to be a very effective method for the separation of small peptides and amino acids.

5.13 Colorimetry and Spectrophotometry

Coloured substances tend to absorb certain wave lengths of light and reflect or transmit remaining portion of it. The light reflected or transmitted gives the colour of that substance. The colourless substances have also the property of absorbing certain wavelengths of invisible light. The wavelength at which a particular substance has maximum absorption of the light energy is designated by the symbol λ_{max}. The values of λ_{max} of some important compounds have been given in Table 5.2.

Table 5.2
λ_{max} OF SOME IMPORTANT COMPOUNDS

Compounds	λ_{max}nm	*Compounds*	λ_{max}nm
Proteins	210 and 280	NAD(P)H	340
Pyrimidines	243	Carotenoids	440
Pyridines	250	Chlorophyll *a*	663
Nucleotides	260	Chlorophyll *b*	649
Vitamin A	325	Acetylene	2105–2150

The basic principle underlying absorption of visible or invisible light finds uses in the measurement of the low concentrations of substances. Different types of instruments are available for measuring the absorption of light by coloured or colourless substances. These, are photometers, absorptiometers, colorimeters, spectrometers, and spectrocolorimeters. The principle underlying

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all these is that the amount of light absorbed by the solution of unknown concentration is compared to that of standard solution and the amount in unknown sample is found out.

Theory : If a beam of light is allowed to pass though a solution, a part of the radiation gets reflected by the walls of the container and the solution, a part gets absorbed and rest gets transmitted. The reflection, absorption or transmission depend upon certain principles. According to Bouger's or Lambert's law, "if a beam of monochromatic light is allowed to pass through a homogeneous absorbing medium, each layer of medium decreases the intensity of light by a constant fraction."

This may be put mathematically as follows :

$$\log_e \frac{I_0}{I} = Kd \qquad ...(i)$$

or

$$I = I_0 e^{-Kd} \qquad (ia)$$

where I represents the intensity of transmitted light,

I_0 the intensity of incident light,

K a constant, and

d the thickness of absorbing medium in cm.

It implies that transmitted light will decrease exponentially as the thickness of absorbing medium tends to increase.

According to Beer' law, "if the absorbing substance is in the gaseous state or dissolved in a transparent solvent, the absorption has been found to be proportional to the molecules of absorbing substance (concentration) in the light path." Hence

$$\log_e \frac{I_0}{I} = Kc \qquad ...(ii)$$

or

$$I = I_0 e^{-Kc} \qquad (iia)$$

where *c* denotes the concentration of substance in g per liter. On combining equations (*i*) and (*ii*), we get

$$\log_e \frac{I_0}{I} = Kcd \qquad ...(iii)$$

As $\log_e \frac{I_0}{I}$ is the logarithm of the reciprocal of transmittance, it is termed as absorbance or extinction coefficient of the medium and denoted by symbol A or E.

Hence equation (*iii*) may be put as follows

$$A = Kcd$$

This equation gives the mathematical expression of the Lambert-Beer's law. From this law, it follows that "the absorbance of a homogeneous medium is equal to product of the absorptivity, optical path length and the concentration of the medium."

K (absorptivity) has been found to depend upon the nature of the substance and is constant for a particular substance. When *d* is 1 cm and *c* is in mol per liter then this constant is termed as molecular *extinction coefficient* (E%/1 cm). The extinction coefficient of NADH at 340 nm is 6.22×10^6 cm^2 per mole.

As K is constant for particular substance, and *d* (length of the optical path) is also constant for an instrument, the absorbance measured has been found to be directly proportional to the

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concentration of the substance. A graph between the absorbance and concentration of substance will be linear.

All the substances, however, do not obey Lambert-Beer's law as well as do not give a linear relationship between concentration of the substance and the absorbance. Association of molecules in the solution and interaction between solute and solvent molecules have been found to exhibit deviation from this law. It therefore becomes essential to study the absorption of light by a solution at various concentrations for testing the obedience to the law.

Experimental : Various types of instruments are available for measuring absorbance. A typical spectrophotometer possesses the following essential components :

(*i*) Source of light,

(*ii*) Monochromator unit,

(*iii*) A detector for light transmitted,

(*iv*) Meter to read the transmission.

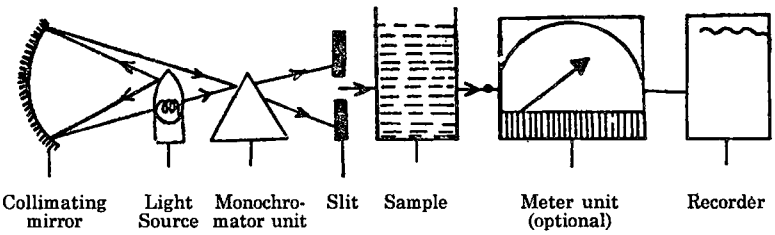

Fig. 5.32. Essential components of a spectrophotometer.

Sequence of these components is depicted in Fig. 5.32. The light source for working in the visible, near infra-red and near ultra violet region is incandescent lamp having tungsten filaments. Hydrogen or deuterium lamps are used for ultra-violet regions. In many spectrophotometers, the light is made parallel by using a collimating lens or a mirror before it is passed on to the monochromator unit which is normally made up of a rotating prism and a filter with a slit. Prism resolves light into different wave bands and the filter permits light of certain wave lengths. The opening of the filter is adjustable. It can be made extremely narrow to obtain resolution upto about 0.5 nm in some spectro-photometers. The instrument has a sample holder which is held between the monochromator unit and the detector. Sample tubes are made up of glasses or fused silica with about 1 mm thick wall and 1 cm internal diameter. The detector is a photosensitive device which gives a linear response in the spectral region. It has been associated with an electrical device (meter) for reading the exact amount of light passing through the sample.

Calorimetry or spectrophotometry in the visible range has been found to be most useful for determining many inorganic compounds and pigments. Ultra-violet spectrophotometry has been used for determining mainly some aromatic and heterocyclic organic compounds. Infra-red spectrophotometry is mainly used for gases.

Calorimetry and spectrophotometry are of prime importance in biochemical research. The three important uses are as follows :

(*i*) If the absorbancy index at a specific wavelength is known, the concentration of a compound can be rapidly determined by measuring optical density at that wavelength. As the value of absorbancy index is very large in nucleotides, very small quantities of the absorbing material (2 – 4 μg) can be accurately measured.

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(*ii*) The course of a reaction can be determined by measuring the rate of formation or disappearance of a light absorbing substance. Thus, NADH absorbs strongly at 340 mμ whereas the oxidised form NAD^+ has no absorption at this wavelength. Therefore, reactions involving the production, or utilisation of NADH (NADPH) can be assayed by the technique.

(*iii*) Compounds can be frequently identified by determining the characteristic absorption spectra in the ultraviolet and visible regions of the spectrum.

5.14 Radiation Techniques

Radiation techniques are based upon properties of certain isotopic elements which emit radioactive rays such as α-rays, β-rays and γ-rays. Like X-rays, these rays could penetrate and affect photographic plates. The elements which emit these rays are called isotopes.

Some isotopes disintegrate because particles break off. β-*particles* are electrons; α-*particles* are helium nuclei (two protons and two neutrons); γ-*rays* are short wavelength radiation. Such isotopes are said to be unstable or *radioisotopes*. Radioactivity can be detected by devices such as *scintillation* and *Geiger counters*. The former are especially useful since they are particularly sensitive to β-particles emitted by most biologically-important isotopes. β-particles cause a liquid (scintillator) in the instrument to produce small flashes of light. The light is measured by meter, which can be scaled to show the amount of radioactivity.

Isotopes can be supplied as chemical precursors to trace out biochemical pathways. For example, if $^{14}CO_2$, radioactive *first* will presumably be in *earlier* steps in a biochemical pathway than substances which become radioactive *later*. This technique is called *labelling*, and $^{14}CO_2$ is described as a *tracer*. Radioisotopes can also label structures. They cause the silver in a photographic film to precipitate as though the film had been made radioactive can thus be identified by the image they form when laid against photographic film. This technique is called *autoradiography*.

Some isotopes are not radioactive and cannot therefore be detected by Geiger and Scintillation counters. Instead, instruments which separate them on account of their mass, such as *mass spectrometers* and *ultracentrifuge*, must be used. Non-radioactive or *stable isotopes* have also been in biological research. $H_2{}^{18}O$, for example, was used to demonstrate that oxygen produced during photosynthesis came from water, not from CO_2, while ^{15}N-thymidine was used to demonstrate semi-conservative replication of DNA.

Experiment : Radioactive isotopes have been found to be more useful than stable isotopes in understanding the mechanism of reactions as well as processes. In biochemistry, the following two techniques find extensive use in understanding the mechanism of processes.

1. Tracer technique : When a biochemist is studying metabolic transformation of a particular substance, his main objective is to observe the fate of that substance *in vivo* under such experimental conditions which cause minimum physiological disturbance to the test organism. Isotopic tracer technique has been found to be most suitable for such types of studies.

In radioactive tracer technique one or more of the atoms in metabolite under study is 'labelled' by means of one of the rare or artificially produced isotopes and its path is followed while frequently testing intermediary compounds at intervals by using sensitive radioactivity detection techniques. By determining precisely the amount of radioactivity in different products the rates and order of their synthesis may be known.

This techniques is now-a-days used for determining the permeability of cells and cell organelles also. Cell or organelles under testing are exposed for a given period to solutions having radioactive

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isotopes of sodium or potassium. After sometime, the cells are centrifuged and the radioactivity in the cells is measured by using a Geiger-Muller counter. By changing the exposure time to solutions having radioactive elements and measuring their levels inside the cell it becomes possible to measure the rate of uptake of ions. Further, it is possible to measure the rate of loss of ions from the cell by putting these cells in a medium having non-radioactive (cold) element.

The Geiger Muller Counter : Its working is based upon the ionizing properties of radioactive rays. The line diagram for the various components of this apparatus is shown in Fig. 5.33. In order to measure radioactivity the substance or the cell extract suspected to contain radioactivity is put in the sample tube which is kept in the holder close to the mica window. Radioactive emission coming from the sample causes ionization of the gas molecules (alcohol vapour + argon) present in the copper tubes of the apparatus. This produces a flash of light in a neon tube and it is recorded over mechanical register.

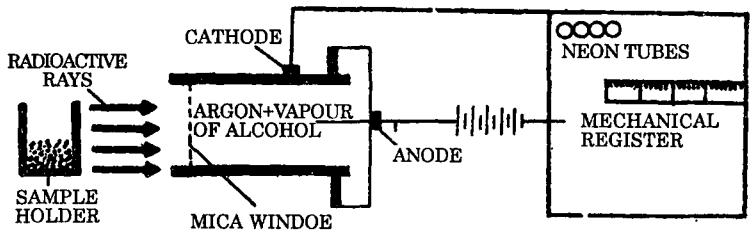

Fig. 5.33. Components of a Geiger-Muller counter.

The conventional G.M. counter is being replaced by scintillation counter or scintillation spectrometer in the modern biochemistry laboratories. Scintillation counter is somewhat more efficient detector for the low energy radiation.

2. Autoradiography : This technique is based upon the ability of the radioactive radiations to blacken a photographic emulsion. After separating a mixture into its components by paper chromatography, the chromatogram is kept in contact with a photographic film in a dark room. When the film is subsequently developed, the radioactive spots on the film appear as blackened spots. The degree of blackening furnishes an idea of the radioactivity present. This technique was used successfully by Melvin Calvin and others in 1950-51 for investigating the pathway of synthesis of carbohydrates from CO_2 in the dark reaction of photosynthesis.

Autoradiography is also being used to determine locations of compounds in a cell or a tissue. For instance, thymidine having ^3H isotope of hydrogen is supplied to actively metabolising cells or tissues for a short duration. The cells are made to count on a slide and fixed. The slide is now kept in contact with a sensitive photographic film. Now this film is developed when we see black spots corresponding to the locations of radioactive materials in the cells. If the black spots are seen over the nucleus, then it can be concluded that thymidine has been incorporated into nuclear contents.

5.15 Applications of Radioisotopes in the Biological Sciences

Investigating Aspects of Metabolism

Metabolic Pathways : Radioisotopes are frequently used for tracing metabolic pathways. This usually involves adding a radioactive substrate, taking samples of the experimental material at various times, extracting and chromatographically, or otherwise, separating the products. Radioactivity is then located either by using a Geiger-Muller chromatogram scanner or by obtaining an autoradiogram of the chromatogram. By identifying the labelled compounds, counting the radioactivity in each of them and plotting suitable graphs, it is possible to obtain considerable

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information on the metabolic pathways involved. Radioactivity detectors can also be attached to GLC or HPLC chromatographic columns to monitor radioactivity coming off the columns during separation.

If it is suspected that a particular compound is metabolised by a particular pathway, then radioisotopes can also be used to confirm this. For instance, it is possible to predict the fate in individual carbon atoms of ^{14}C-acetate through the tricarboxylic acid cycle (TCA cycle). Methods have been developed whereby intermediates of the cycle can be isolated and the distribution of carbon within each intermediate can be ascertained. This is the so-called *specific labelling pattern*. Should the actual pattern coincide with the theoretical pattern then this is very good evidence for the operation of the TCA cycle.

Another example of the use of radioisotopes to confirm the operation, or otherwise, of a metabolic pathways is in studies carried out on glucose catabolism. There are numerous ways whereby glucose can be oxidised, the two most important ones in aerobic organisms being glycolysis followed by TCA pathway and the pentose phosphate pathway. Frequently organisms or tissues possess the necessary enzymes for both pathways to occur and it is of interest to establish the relative contribution of each to glucose oxidation. Both pathways involve the complete oxidation of glucose to carbon dioxide, but the origin of the carbon dioxide in terms of the six carbon atoms of glucose is different (at least in the initial stages of respiration of exogenously added substrate). Thus it is possible to trap the carbon dioxide evolved during the respiration of specially labelled glucose (*e.g.*, ^{14}C-6-glucose in which only the C-6 atom is radioactive and ^{14}C-1-glucose) and obtain an evaluation of the contribution of each pathway to glucose oxidation.

The use of radioisotopes in studying the operation of the TCA cycle in evaluating the pathway of glucose catabolism is an example of how they can be used in confirming metabolic pathways.

Metabolic turnover times : Radioisotopes provide a convenient method of ascertaining turnover times for particular compounds. As an example, the turnover of proteins in rats will be considered. A group of rats is injected with a radioactive amino acids and left for 24 hours, during which time most of the amino acid will be assimilated into proteins. The rats are then sacrificed at suitable time intervals and radioactivity in organs or tissues of interest is determined. In this way it is possible to ascertain the rate of metabolic turnover of protein. Using this sort of method it has been shown that liver protein is turned over in 7 to 14 days, while skin and muscle protein is turned over every 8 to 12 weeks and collagen is turned over at a rate of less than 10% per annum.

Studies of absorption, accumulation and translocation : Radioisotopes have been very widely used in studying the mechanisms and rates of absorption, accumulation and translocation of inorganic and organic compounds by both plants and animals. Such experiments are generally simple to perform and can also yield evidence on the route of translocation and sites of accumulation of molecules of biological interest.

Pharmacological studies : Another field where radioisotopes are widely used is in the development of new drugs. This is a particularly complicated process since, besides showing if a drug has a desirable effect, much more must be ascertained before it can be used in the treatment of clinical condition. For instance, the site of drug accumulation, the rate of accumulation, the rate of metabolism and the metabolic products must all be determined. In each of these areas of study, radiotracers are extremely useful, if not indispensable. For instance, autoradiography on whole sections of experimental animals yields information on the site and rate of accumulation while typical techniques used in metabolic studies can be used to follow the rate and products of metabolism.

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5.17 Analytical Applications

Isotope dilution analysis : There are many compounds present in living organisms which cannot be accurately assayed by conventional means, since they are present in such low amount and in mixtures of similar compounds. Isotope dilution analysis offers a convenient and accurate way of overcoming this problem and avoids the necessity of quantitative isolation. For instance, if the amount of iron in a protein preparation is to be determined, this may be difficult using normal methods, but it can be done if a source of ^{59}Fe is available. This is mixed with the protein and a sample of iron is subsequently isolated, assayed for total iron and the radioactivity determined.

If the original specific activity was 10000 d min^{-1} per 10 mg and the specific activity of the isolated iron was 9000 d min^{-1} 10 mg then the difference is due to the iron in the protein (x), *i.e.*,

$$\frac{9000}{10} = \frac{10000}{10 + x}$$

and

$$x = 1.1 \text{ mg}$$

This technique is widely used in, for instance, studies on trace elements.

Radioimmunoassay : One of the most significant advances in biochemical techniques in recent years has been the development of radioimmunoassay (RIA) (see 5.17).

Radiodating : A quite different analytical use for radioisotopes is in the *dating* (*i.e.*, determining the age) of rocks, fossil and sediments. In this technique it is assumed that the proportion of an element which is naturally radioactive has been the same throughout time. At the time of fossilisation or deposition the radioactive isotope begins to decay. By determining the amount of radioisotope remaining (or by examining the amount of a decay product) and from a knowledge of the half life, it is possible to date the sample. For instance, if the radioisotope normally composes 1% of the element and it is found that the sample actually contains 0.25% then two half lives can be assumed to have elapsed since deposition. If the half life is 1 million years then the sample can be dated as being 2 million years old.

For long term dating, isotopes with long half lives are necessary, such as ^{235}U, ^{238}U and ^{40}K, whereas for shorter term dating ^{14}C is widely used. It cannot be over-emphasised that the assumptions made in radiodating are sweeping and hence palaeontologists and anthropologists who use this technique can only give approximate dates to their samples.

Enzyme and ligand binding studies : Virtually any enzyme reaction can be assayed using radiotracer methods as outlined earlier provided that a radioactive form of the substrate is available. Radiotracer-based enzyme assays are more expensive than other methods, but frequently have the advantages of a higher degree of sensitivity. Radioisotopes have also been used in studying the mechanism of enzyme action and in ligand binding studies.

5.17 Radioimmunoassay

Radioimmunoassay is one of the most important techniques in the clinical and biochemical fields for the quantitative analysis of hormones, steroids and drugs. It combines the specificity of the immune reaction with the sensitivity of radioisotope techniques. Alternative names used for RIA include *saturation analysis, displacement analysis and competitive radioassay.*

The technique is based on the competitions between unlaballed antigen and a finite amount of the corresponding radio-labelled antigen for a limited number of antibody binding sites in a fixed amount of antiserum. At equilibrium in antigen excess there will be both free antigen and

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antigen bound to the antibody. Under standard conditions the amount of labelled antigen bound to antibody will decrease as the amount of unlablled antigen in the sample increases, for example :

$$4Ag^* + 4Ab \rightleftharpoons 4Ag^*Ab$$

$$4Ag + 4Ag^* + 4Ab \rightleftharpoons 2Ag^*Ab + 2AgAb + 2Ag^* + 2Ag$$

$$12Ag + 4Ag^* + 4Ab \rightleftharpoons Ag^* + Ab + 3AgAb + 3Ag^* + 9Ag$$

where Ab, Ag, Ag* and AgAb represent one equivalent of antibody, unlabelled antigen, labelled antigen and antigen-antibody complex respectively.

By using known amounts of unlabelled antigen and a fixed amount of antibody and labelled antigen, the amount of labelled antigen bound as a function of the total antigen added is measured and a calibration curve is constructed (Fig. 5.34). This calibration curve may then be used to determine the amount of antigen in samples treated similarly.

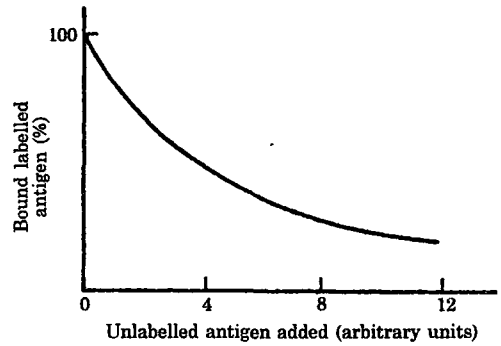

Fig. 5.34. A typical radioimmunoassay calibration curve.

The major advantages of RIA include :

(*i*) the ability to assay any compound that is immunogenic, available in pure form, and can be radio-labelled;

(*ii*) Its high sensitivity—some compounds may be detected at a level of pg cm^{-3};

(*iii*) its high specificity;

(*iv*) its precision which is comparable to that of other physico-chemical techniques and far better than bioassays;

(*v*) its ease of automation so that a minimum of manual handling and data processing is necessary. This allows a large number of samples to be processed at minimal cost.

The major disadvantages of RIA include :

(*i*) the relatively high cost of equipment and reagents. Gamma scintillation counters are expensive to buy and maintain, and radioiodine is not a cheap reagent;

(*ii*) the short shelf-life of reagents—the half lives of ^{125}I and ^{131}I are 60 days and 8 days respectively, necessitating relatively frequent labelling of antisera;

(*iii*) the radiological hazards of using radioiodine, particularly during the labelling of antisera, which must be respected fairly regularly. Staff should have regular thyroid scans and be rested if the level of radioactivity increases significantly;

(*iv*) assays usually take days rather than hours.

5.17 Immunoradiometric Assay (IRMA)

This utilises purified radio-labelled antibody and is often more sensitive than RIA, for example thyroid stimulating hormone (TSH) may be assayed by allowing it to bind to an antibody complementary to the α-subunit of TSH attached to Sephadex and then reacting it with radio-labelled antibody against the TSH subunit (Fig. 5.35).

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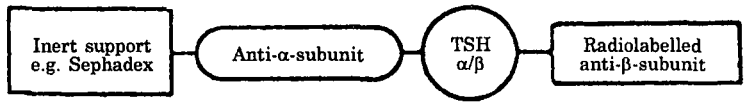

Fig. 5.35. The immunoradiometric assay of thyroid stimulating hormone.

5.18 Polarimetry

Introduction : When certain organic liquids, solutions (like sugar) or quartz crystals are placed in the path of plane polarized light, the plane of plarisation is rotated. The property by virtue of which the plane of polarisation of light is rotated is called optical activity and the substances possessing this property are said to be optically active. Substances which rotate the plane of polarized light toward the right (clockwise) are called dextro rotatory (+) while which rotate towards left (anticlockwise) are called laevo rotatory (–)]. A mixture of these two varieties in equal proportions will be optically inactive and is called racemic form.

Theory

According to wave theory of light, an ordinary ray of light is considered to be vibrating in all planes at right angles to the direction of its propagation. If this ordinary ray of light is passed through a Nicol prism (a special prism obtained by combining two prisms of iceland spar suitably cut), the emergent ray has its vibrations only in one plane. This light having wave motion in only one plane is known as plane polarized light (Fig. 5.36).

It has been found that the magnitude of the rotation depends upon the following factors :

(*i*) Nature of the substance.
(*ii*) Length of the liquid column (*l*) through which light passes.
(*iii*) Concentration of the solution.
(*iv*) Nature of the solvent.
(*v*) Temperature of the solution (*t*).
(*vi*) Wavelength of the light used.

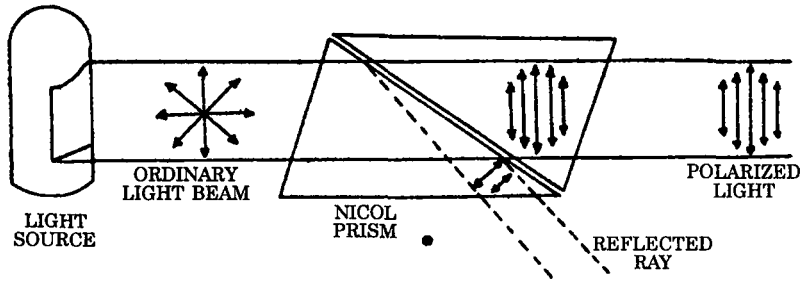

Fig. 5.36. Plane Polarized light.

The rotatory power of a given solution is generally expressed as specific rotation, $[\alpha]^t_D$. It is the number of degrees of rotation of the plane polarized light produced by one decimeter in length filled with a solution having one gram of the substance per ml. The measurement is carried out at a temperature *t* using sodium light. The specific rotation can be calculated by using the following relation :

$$[\alpha]_D^t = \frac{100 \times \text{observed angle of rotation}}{\text{length in decimetres} \times \text{Grams of substance in 100 ml of solution}}$$

or $$[\alpha]_D^t = \frac{100 \times \theta}{l \times c}$$

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It should be kept in mind that for very dilute solutions in any given non-active solvent, the specific rotation is constant.

Specific rotation may be defined as the rotations in degrees of a solution having 1 g of material in a 10 cm (1 dm) polarimeter tube.

The optical rotation of the compounds has been found on vary according to the wave length of light used. This variation is termed as *optical rotatory dispersion.*

Apparatus : The various components of a polarimeter are depicted in Fig. 5.36. The light source is generally the sodium lamp which is emitting light at 589 nm (D line of sodium). The light is made to filter through a slit and rendered parallel through a lens. Then it is allowed to pass on to a nicol prism which functions as a polariser. The sample tube (polarimeter tube) is generally 1 dm long. The light coming out through the tube is allowed to pass through another nicol prism which functions as analyser. The entire system is put inside a tube. When there is no optically active sample in polarimeter tube, the lens on analyser side is so adjusted that the intensity of light seen on the viewer side (eye) becomes minimum. When there is an optically active compound in the tube, the plane of light gets rotated. The analyser side lens is rotated in order to minimise the light intensity again. This lens is fitted on a scale and the angle of desired rotation can be known from that scale. If we know this absorbed rotation in degrees, the specific rotation can be calculated as given earlier. If the analysing lens is rotated clockwise, the substance is called dextro-rotatory (+). If the lens is rotated counter-clockwise, the substance is called laevo-rotatory (–).

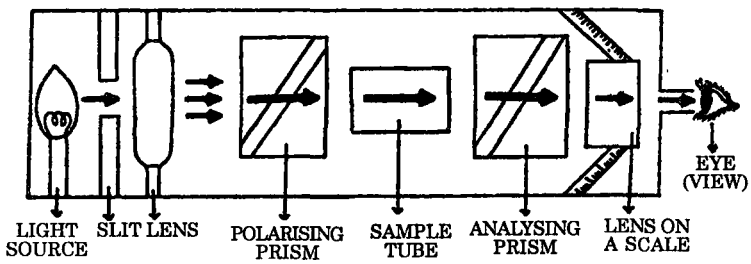

Fig. 5.36. Various components of a polarimeter.

Uses : Polarimetry is mainly employed for identifying unknown compounds and also for knowing concentration of known compounds. It can also be employed for studying an enzymatic reaction in which the substrate and product are having different optical rotations. Optical rotatory dispersion measurements can furnish some useful information about the atomic organisation of a molecule.

Quantitative methods of analysis by polarimetry are widespread particularly for plant control in the pharmaceutical industry. The most extensive application, however, is in the sugar industry.

In the absence of other optically active substance sucrose can be determined directly by measuring the angle of rotation, $[\alpha]_D^{20}$ being equal to +66.5°. If other active substances are present, it is necessary to measure the optical rotation before and after hydrolysis of the sugar solution. Sucrose is the only common sugar to undergo hydrolysis in the presence of acid the resultant dextrose and levulose mixture has an optical rotation of –20.2°. The amount of sucrose present is calculated from the difference in rotation before and after inversion.

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6 Chemistry of Carbohydrates

6.1 Introduction to Carbohydrates

Carbohydrates are among the most widely distributed compounds in both plant and animal kingdoms. Plants can build up carbohydrates from carbon dioxide by *photosynthesis*. Many plants and animals contain large quantities of carbohydrates as *reserve food material*. Carbohydrates are also important *structural components*.

The name 'carbohydrate' arose from the mistaken belief that *substances of this kind were hydrates of carbon, since the molecular formula of many carbohydrates could be expressed in the form* $C_x(H_2O)_y$, for example, glucose. ($C_6H_{12}O_6$), sucrose ($C_{12}H_{22}O_{11}$) etc. It should be kept in mind that all organic compounds containing hydrogen and oxygen in the proportion found in water are not carbohydrates. For example, formaldehyde HCHO, for the present purpose written as $C(H_2O)$, acetic acid CH_3COOH written as $C_2(H_2O)_2$ and lactic acid $CH_3CHOHCOOH$ written as $C_3(H_2O)_3$ are not carbohydrates. Also a large number of carbohydrates such as rhamnose ($C_6H_{12}O_5$), are known which do not contain the usual proportions of hydrogen to oxygen. Finally, certain carbohydrates are also known which contain nitrogen or sulphur in addition to carbon, hydrogen and oxygen.

Simple carbohydrates are also known as *sugars* or *saccharides* (L. Saccharum, sugar) and the ending of the names of most sugars is *ose*. Thus, we have such names as sucrose for ordinary table sugar, glucose for blood sugar, maltose for sugar, and so on.

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From the above discussion it can be concluded that the definitions described above are *not* correct. However, *carbohydrates are now defined chemically as aldehyde or ketone derivatives of the higher polyhydric (more than one OH group) alcohols or as compounds which yield these derivatives on hydrolysis.*

6.2 Classification of Carbohydrates

The carbohydrates are divided into four major classes depending upon whether or not they undergo hydrolysis, and if they do, on the number of products formed.

1. Monosaccharides : The term 'monosaccharides' is employed for such sugars that on hydrolysis yield, no further, lower sugars. The general formula of monosaccharides is $C_nH_{2n}O_n$. The monosaccharides are subdivided as trioses, tetroses, pentoses, hexoses or heptoses, depending upon the number of carbon atoms they possess; and as *aldoses* or *ketoses,* whether the *aldehyde* or *ketone* groups are present.

One of the methods of naming relates to the last point. Thus, glyceraldehyde may be termed as an aldotriose, and dihydroxyacetone is then called a ketotriose. Among the common ketoses, or ketonic monosaccharides, the carbonyl oxygen is found on the C-2 carbon atom (adjacent to the uppermost one).

Generic names for the ketoses are formed by insertion of "ul", thus; pentulose, heptulose, and so on.

Some examples of monosaccharides are given in Table 6.1.

Table 6.1
GENERAL NOMENCLATURE OF THE MONOSACCHARIDES

Number of Carbons	*Functional group*	
	Aldehydes (aldoses)	*Ketones (ketoses)*
3	a triose (aldotriose) CHO—CHOH—CH_2OH *Glycerose*	a triulose (ketoriose) CH_2OH—CO—CH_2OH *Dihydroxyacetone*
4	a tetrose (aldotetrose) CHO—$(CHOH)_2$—CH_2OH *Erythrose*	a terulose (ketotetrose) CH_2OH—CO-CHOH—CH_2OH *Erythrulose*
5	a pentose (aldopentose) CHO—$(CHOH)_3$—CH_2OH *Ribose*	a Pentulose (ketopentose) CH_2OH—CO—$(CHOH)_2$—CH_2OH *Ribulose*
6	a hexose (aldohexose) CHO—$(CHOH)_4$—CH_2OH *Glucose*	a hexulose (ketohexose) CH_2OH—CO—$(CHOH)_2$—CH_2OH *Fructose*

Most carbohydrates are referred to by their common names rather than by systematic names.

2. Oligosaccharides : Carbohydrates that hydrolyse to yield 2 to 10 moles of a monosaccharide are sometimes called oligosaccharides. The general formula is $C_n(H_2O)_{n-1}$.

Carbohydrates that undergo hydrolysis to produce only two moles of a monosaccharide are called *disaccharides* (*e.g.*, sucrose and maltose, both of which have same molecular formula $C_{12}H_{22}O_{11}$); those they yield three moles of a monosaccaride are called *trisaccharides* (*e.g.*, raffinose which has molecular formula, $C_{18}H_{32}O_{16}$); and so on.

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$$\underset{\text{Sucrose}}{C_{12}H_{22}O_{11}} + H_2O \xrightarrow{H^+} \underset{\text{Glucose}}{C_6H_{12}O_6} + \underset{\text{Fructose}}{C_6H_{12}O_6}$$

$$\underset{\text{Maltose}}{C_{12}H_{22}O_{11}} + H_2O \xrightarrow{H^+} 2\,\underset{\text{Glucose}}{C_6H_{12}O_6}$$

$$\underset{\text{Raffinose}}{C_{18}H_{32}O_{16}} + 2H_2O \xrightarrow{H^+} \underset{\text{Glucose}}{C_6H_{12}O_6} + \underset{\text{Fructose}}{C_6H_{12}O_6} + \underset{\text{Galactose}}{C_6H_{12}O_6}$$

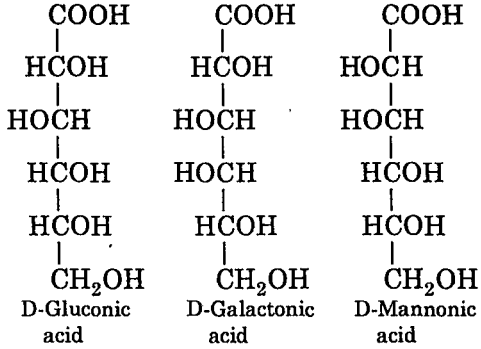
3. Polysaccharides : Carbohydrates that yield a large number of moles of monosaccharides, i.e., > 10, are known as polysaccharides. The general formula of polysaccharides is $(C_6H_{10}O_5)_n$. Starch and cellulose are examples of polysaccharides; they are both glucose polymers. Hydrolysis of either starch or cellulose yields a large number of glucose units.

$$\underset{\substack{\text{Starch or}\\ \text{cellulose}}}{(C_6H_{10}O_5)_n} + nH_2O \xrightarrow{H^+} \underset{\text{Glucose}}{nC_6H_{12}O_6}$$

Polysaccharides are very long chains or polymers, of monosaccharides that may be either linear or branched in structure.

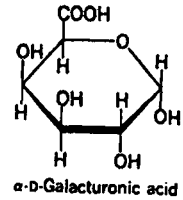
Alternative Classification of Carbohydrates

Carbohydrates may be classified as either reducing or non-reducing sugars. The reducing sugars, which are more common, are able to function as reducing agents because free, or potentially free, aldehyde groups, as in the cyclic hemiacetal forms, are present in the molecule. This aldehyde group is readily oxidized to the carboxylic acid at neutral pH by mild oxidizing agents and enzymes. This property is utilized in detecting and quantitating monosaccharides, especially glucose, in biological fluids such as blood or urine. The monocarboxylic acid that is formed is known as an aldonic acid (*e.g.*, gluconic acid from glucose). The structures of several of these are shown.



In the presence of a strong oxidizing agent like HNO_3, both the aldehyde and the primary alcoholic function will be oxidized to yield the corresponding dicarboxylic or aldaric acid (*e.g.*, galactaric acid).

One of the more important oxidation products of monosaccharides is the monocarboxylic acid obtained by the oxidation of only the primary alcoholic group, usually by specific enzymes, to yield the corresponding uronic acid (*e.g.*, galacturonic acid). Such acids are components of important heteropolysaccharides found in nature.



α-D-Galacturonic acid

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The aldehyde and ketone groups of monosaccharides may be reduced non-enzymically (with hydrogen or $NaBH_4$) or with enzymes to yield the corresponding sugar alcohols. Thus, D-glucose when reduced yield D-sorbitol, and D-mannose produces D-mannitol. Sorbitol is found in the berries of many higher plants, especially in the *Rosaceae*; it is a crystalline solid at room temperature but has a low melting point. D-Mannitol is found in algae and fungi. Both compounds are soluble in H_2O and have a sweet taste.

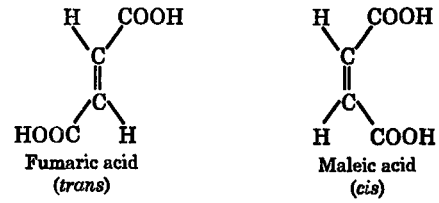
Nomenclature of Carbohydrates

Simple sugars have three-letter abbreviations [*e.g.*, Glc (glucose), Gal (galactose), Man (mannose), Fuc (fucose)]. Sugar derivatives can also be abbreviated, such as GlcNAc (*N*-acetylglucosamine), GalNAc (*N*-acetylgalactosamine). This also allows an abbreviated form of description for sugars that are bonded together and the nature of the covalent bonds. Thus, for example, lactose can be represented as Galβ1→4Glc.

Stereoisomerism in Carbohydrates

The study of carbohydrates requires an understanding of isomerism, especially stereoisomerism. The subject of isomerism may be divided into *structural isomerism* and *stereoisomerism*. Structural isomers have the same molecular formula but differ from each other by having different structures; that is, they differ in the order in which their atoms are bonded together. Stereoisomers have the same molecular formula and the same structure, but they differ in *configuration*, that is, in the arrangement of their atoms in space. Structural isomers, in turn, can be of three types.

The subject of stereoisomerism can be divided into the smaller areas of *optical* isomerism and *geometrical* (or *cis-trans*) isomerism. The latter type of isomerism is illustrated by the *cis-trans* pair, fumaric and maleic acids.



Fumaric acid (*trans*) Maleic acid (*cis*)

Optical Isomerism

This is the type of isomerism commonly found in carbohydrates; it is usually encountered when a molecule contains one or more *chiral* (Greek cheir = hand) or asymmetric carbon atoms. The subject of stereoisomerism was extensively developed after van't Hoff and LeBel introduced the concept of the *tetrahedral carbon atom.*

Optical Activity

Almost all the properties of the two members of an enantiomeric pair are identical : they have the same boiling point, the same melting point, the same solubility in various solvents. They also exhibit optical activity; in this property, they differ in one important manner. One member of the enantiometric pair will rotate a plane of polarized light in a clockwise direction and is therefore said to be *dextro*rotatory. Its mirror image isomer or enantiomer will rotate the plane of polarized light to the same extent, but in the opposite or counterclockwise direction. This isomer is said to be *levo*rotatory. It must be noted, however, that not all compounds possessing a chiral center are chiral or exhibit optical activity. On the other hand, a molecule may possess chirality, exhibit optical activity, and not contain a chiral center.

D-Glyceraldehyde as a Reference Compound

With the existence of a large number of optical isomers in carbohydrates, it is also necessary to have a reference compounds. The simplest monosaccharide that possesses an asymmetric carbon

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atom has been chosen as the reference standard; this compound is the triose *glycerose* or *glyceraldehyde.*

Glyceraldehyde has a single asymmetric carbon atom (the central one) and so two *stereoisomers* (also called *optical isomers*) are possible, that is two forms of glyceraldehyde, denoted as D- and L-*glyceraldehyde*, which are mirror images of each other (Fig. 6.1). Stereoisomers also exist for amino acids.

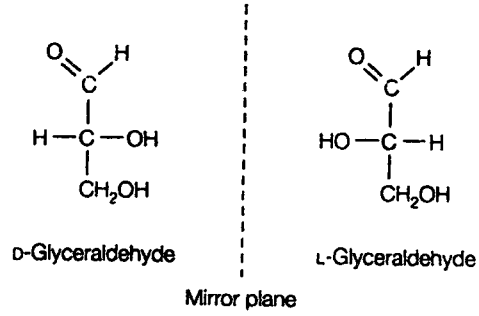

Fig. 6.1. D- and L-glyceraldehyde are mirror images of each other (stereoisomers or optical isomers).

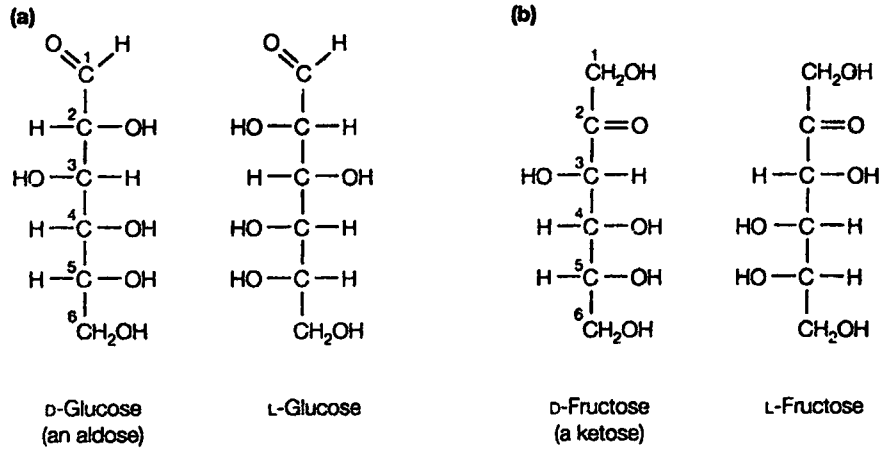

Fig. 6.2. (*a*) D- and L-glucose; (*b*) D- and L-fructose.

Sugars with four, five, six or seven carbons are called *tetroses, pentoses, hexoses* and *heptoses* respectively. In these cases the sugars may have more than one asymmetric carbon atom. The convention for numbering carbon atoms and naming configurations is as follows :

(*i*) the carbon atoms are numbered from the end of the carbon chain starting with the aldehyde or ketone group, which is carbon 1 (C-1);

(*ii*) the symbols D and L refer to the configuration of the asymmetric carbon atom furthest from the aldehyde or ketone group.

Thus, for example, glucose, an aldohexose, exists as D and L Forms [Fig. 6.2 (*a*)]. The furthest asymmetric carbon from the aldehyde group is C-5. D-Glucose [Fig. 6.2 (*a*)] is called D because the configuration of the atoms bonded to C-5 is the same as for D-glyceraldehyde (Fig. 6.1). Similarly D-fructose [a ketohexose; Fig. 6.2 (*b*)] is designated D because the configuration at C-5 matches that for D-glyceraldehyde. D sugars that differ in configuration at only a single asymmetric carbon atom are called *epimers.* Thus D-glucose and D-galactose are epimers, differing only in their configuration at C-4 (Fig. 6.3).

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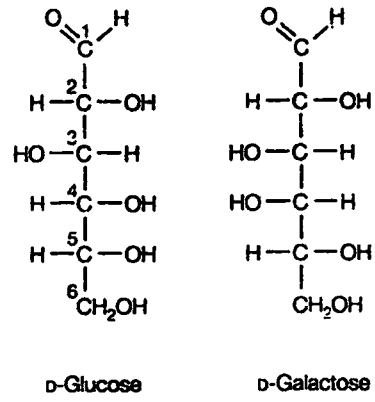

Fig. 6.3. The epimers D-glucose and D-galactose.

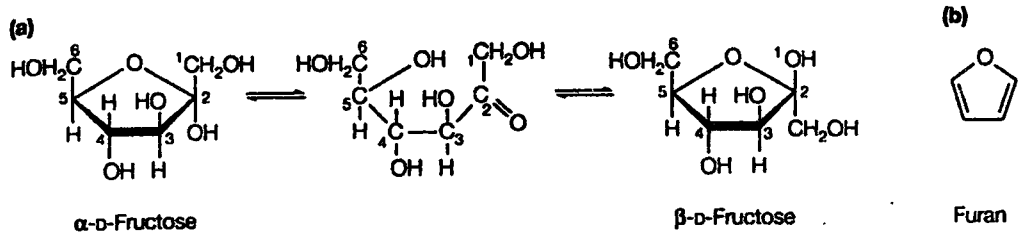

Fig. 6.4. (*a*) Cyclization of the open-chain form of D-fructose; (*b*) the structure of furan.

The pyranose ring of a six-carbon aldose sugar can exist in either a *boat* or a *chair* configuration (Fig. 6.5). The substituents attached to the ring carbons that extend parallel to the symmetry axis are said to be axial (*a*) whilst those that extend outward from this axis are said to be equatorial (*e*) (Fig. 6.5). In the boat form, there is considerable steric hindrance between the various groups attached to the carbon atoms of the ring and therefore this form is less favourable energetically. Hence the chair form predominates, as shown for β-D-glucose in Fig. 6.5, where all the axial positions are occupied by hydrogen atoms.

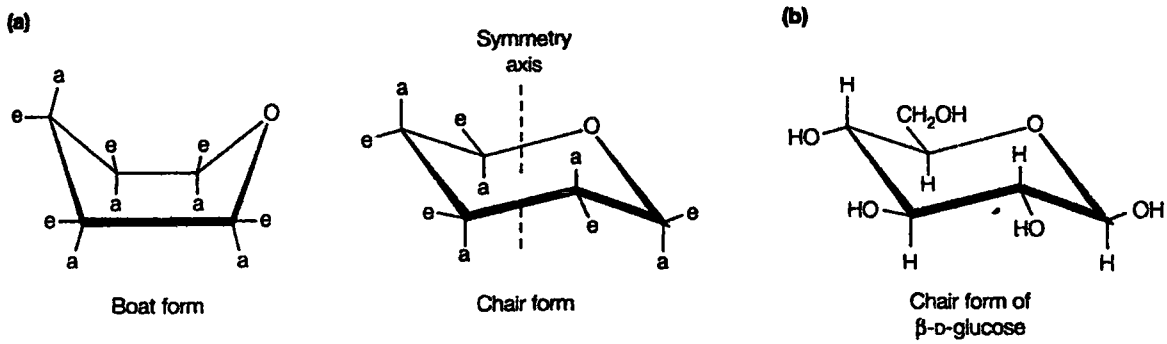

Fig. 6.5. (*a*) Chair and boat conformations of pyranose rings; (*b*) stable chair form of β-D-glucose.

Most of the naturally occurring monosaccharides are members of the D-Family. The D-family of aldoses is depicted in Fig. 6.6.

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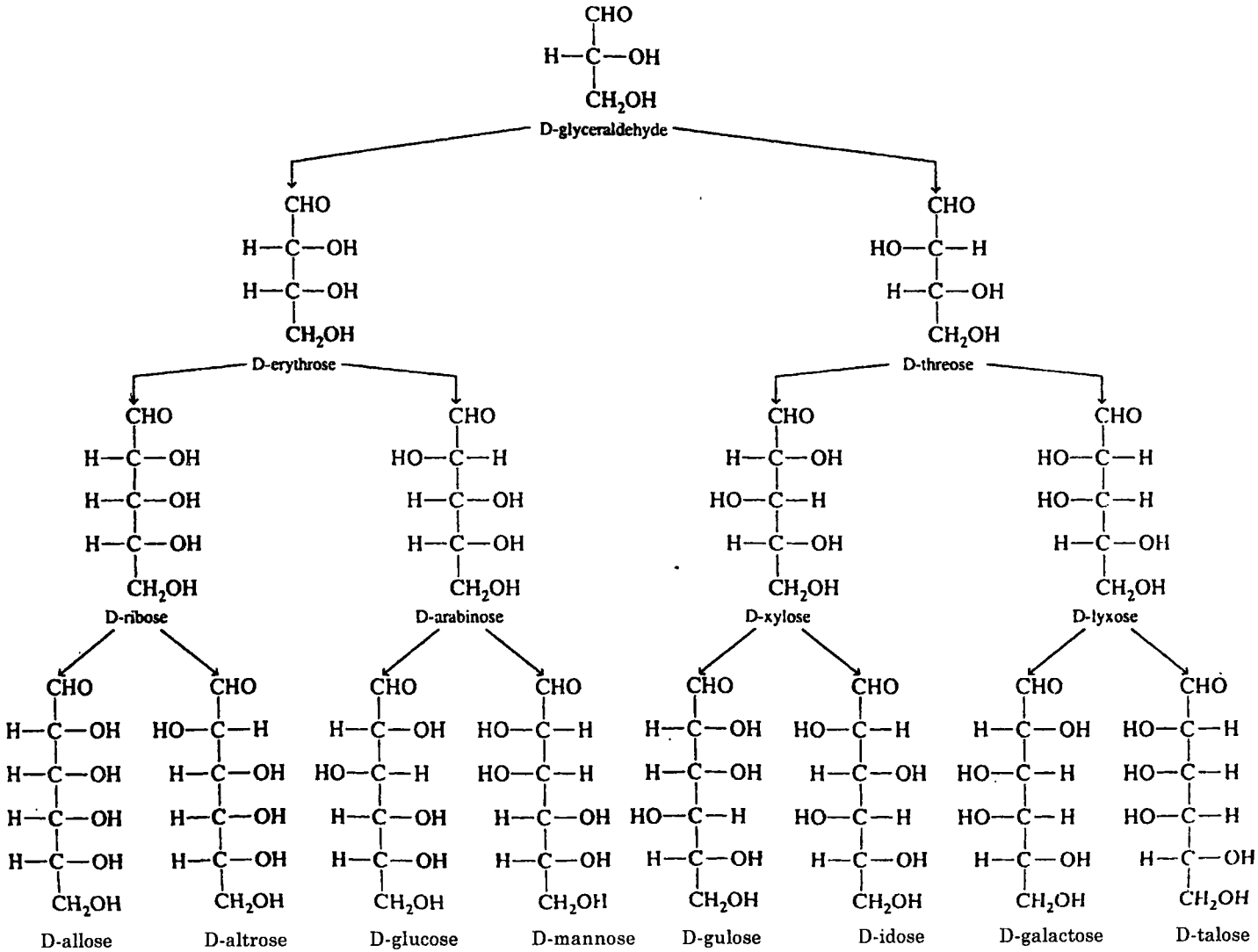

Fig. 6.6. The D-family of aldoses.

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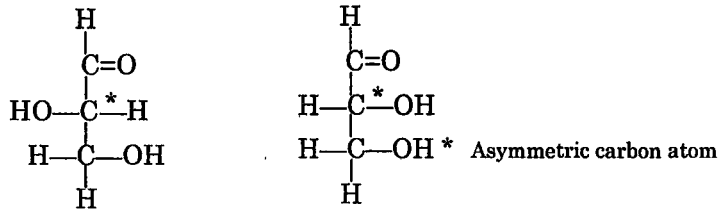
6.3 Properties of Monosaccharides

Physical properties : Monosaccharides are sweet tasting colourless solids, having solubility in water but sparingly soluble in alcohol and insoluble in ether. They have asymmetric carbon atom and therefore exist in different isomeric forms. An example is glyceraldehyde which may exist in two forms which are mirror images of each other.



L–Glyceraldehyde D-Glyceraldehyde

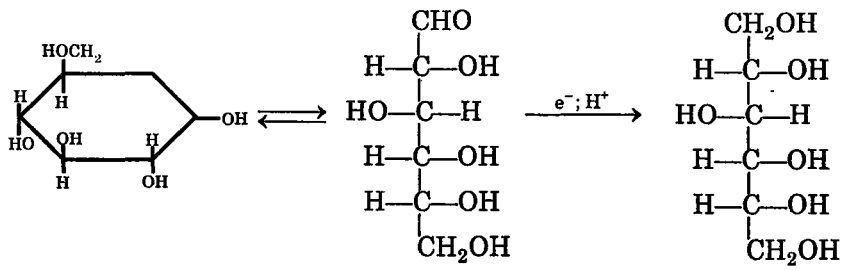
If a polarized light (light vibrating in one plane) is allowed to pass through a solution of these carbohydrates, the plane of the light gets rotated to either right or left. All monosaccharides which have a D glyceraldehyde unit are termed as D-Sugars. L-monosaccharides have a L-glyceraldehyde unit in their structure.

Carbohydrates isolated from living system are not pure compounds, but a mixture of several isomers. The optical rotation may undergo a change due to interconversion of isomers. Fresh solution of glucose yields an optical rotation of +112° which gets changed to +52.7° on standing.

Similarly, fructose yields an optical rotation of –113° which gets changed to –92°. This change in optical rotation is termed as *mutarotation.*

Chemical properties : As monosaccharides are aldehyde or ketone derivatives of the higher polyhydric alcohols, they give most, but not all, of the characteristic reactions of the carbonyl group as well as of the alcoholic group. In addition to these reactions, they also give rise to some special reactions due to the presence of a carbonyl and alcoholic group in the same molecule.

1. Reduction : The nature of the product formed by the reduction of aldoses depends upon the nature of the reagent. For example, D-glucose when reduced yields D-sorbitol and D-mannose produces D-mannitol. Sorbitol is found in the berries of many higher plants especially in the rosacea.



When ketoses are reduced by the reducing agents, a mixture of two alcohols is obtained in each case. For example, D-fructose always yields a mixture of two epimers, D-sorbitol and D-mannitol.

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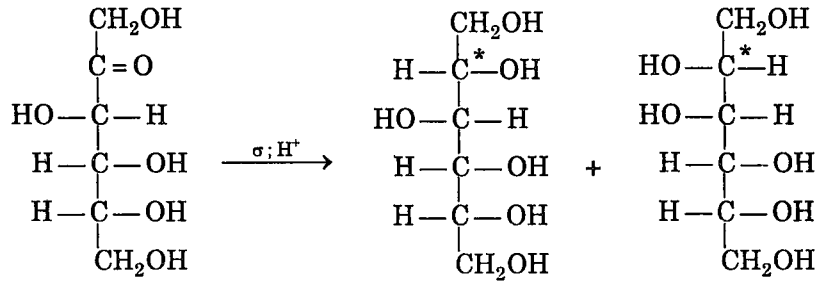
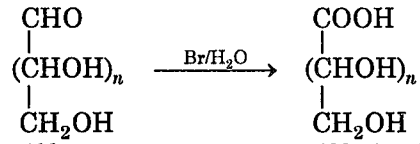


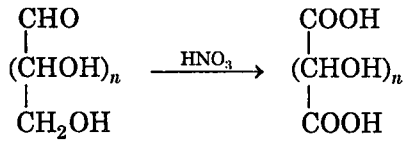
D-Fructose D-Sorbitol D-Mannitol

2. Oxidation : As with reduction, the product of oxidation depends upon the nature of the reagent. Bromine water is a general reagent which selectively oxidises the —CHO group to a —COOH group and brings about conversion of an aldose to an aldonic acid.



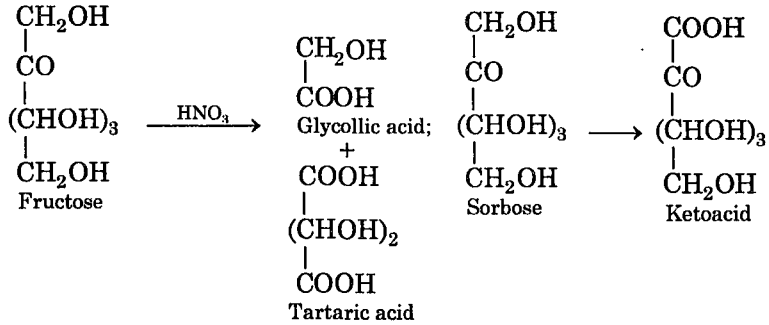
Aldose Aldonic acid

Bromine water cannot oxidise the ketoses. When oxidation of adloses is done with strong oxidising reagents like nitric acid, both the aldehyde and primary alcoholi groups are oxidised to yield dicarboxylic acids known as aldaric acids.



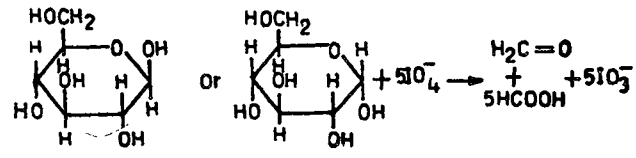
Aldose Aldaric acid

Ketoses, on the other hand, are split up by the action of nitric acid to form a mixture of acids having fewer carbon atoms. However, it has been observed that the carbon chain of sorbose is not ruptured during oxidation with nitric acid but a keto acid is the product.



Fructose Tartaric acid Sorbose Ketoacid

D-Glucose on periodate oxidation yields one mole of formaldehyde and 5 moles of formic acid.



ß-D-Glucopyranose α-Glucopyranose

Similarly, the glycoside methyl α–D-gluco-pyranoside will react with periodate as shown below :

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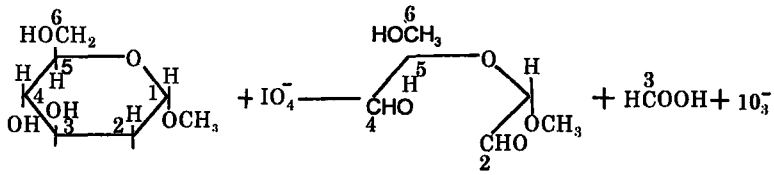


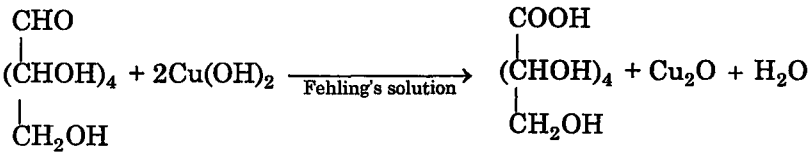
Glycoside
α-D-Gluco pyranoside

Benedict's reagent (an alkaline solution containing a cupric citrate complex) and Tollen's solution [$Ag(NH_3)_2OH$] oxidise and thus give positive tests with aldehydes and with α-hydroxyl ketones. Both reagents, therefore, give positive tests with aldoses and ketoses. Sugars that give positive tests with Tollen's and Benedict's solutions are known as reducing sugars.

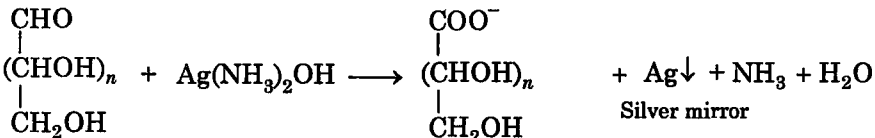
Ag^+ (complex) + Aldose or ketose $\longrightarrow$ Ag + Oxidation
Tollen's reagent (Silver mirror) Products

Cu^{2+} (complex) + Aldose or ketose $\longrightarrow$ Cu_2O + Oxidation
Benedict's solution blue (Brick red ppt) Products

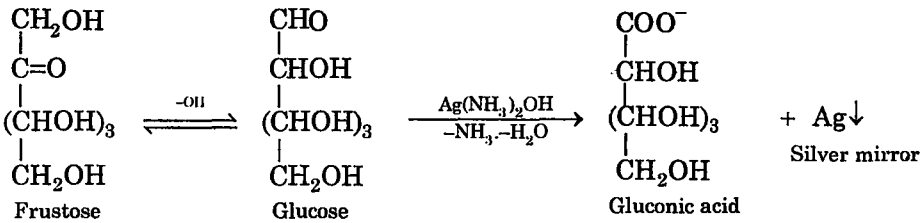
Oxidations with both Benedict's and Tollen's reagents take place in alkaline solution.



Gluconic acid



Aldose — Aldonic acid — Silver mirror



Frustose — Glucose — Gluconic acid — Silver mirror

Fructose (a ketose) is a reducing sugar because of enolization property of the ketonic group. In the open chain form, fructose forms an enol by movement of α-hydrogen (on Cl). When the enol reverts to carbonyl form, an aldose (glucose) may be formed. Thus, ketoses (the α-hydroxyketones), in presence of alkali undergo *ene—diol rearrangement* to aldoses and hence, reduce Fehling's and Tollens reagents.

In general, *all monosacchsarides are reducing sugars* irrespective of aldose or ketose.

3. Ester formation : The formation of sugar esters indicates the presence of alcohol groups. For example, the total number of acyl groups which can thus be taken up by a molecule of the sugar is a measure of the number of such alcohol groups. For instance, the acetylation of glucose results in a pentaacetate, indicating that it (glucose) contain 5 OH groups.

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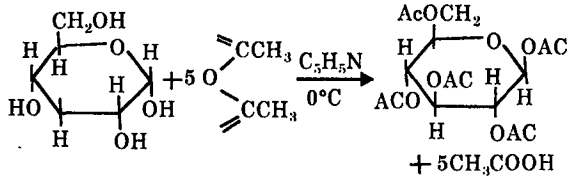
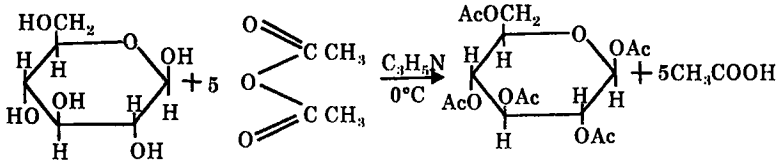
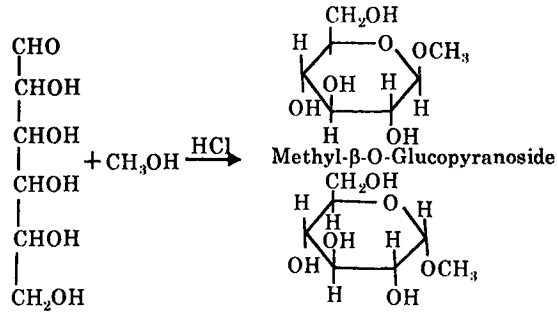


α-D-Glucopyranose Acetic anhydride Penta-O-Acetyl-α-D-Glucopyranose



β-D-Gludopyranose Acetic Anhydride Penta-O-Acetyl-β-D-Glucopyranose

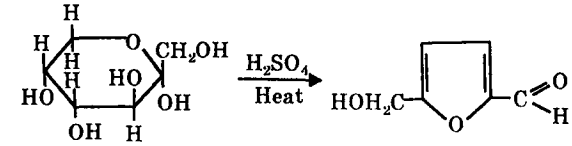
4. Clycoside formation : This is one of the most important properties of monosaccarides. Consider as an example of the formation of the methyl glycoside of glucose. When D-glucose in solution is treated with methanol and HCl, two compounds are obtained.



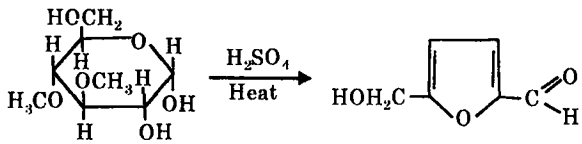
Glucose Methyl-β-O-Glucopyranoside Methyl-α-D-Glucopyranoside

Glucosides are found in many drugs, spices and in the constituents of animal tissues. The glycosides which are important in medicines due to their action on the heart all contain steroids as the aglycone component. The examples of these include derivatives of digitalis and strophanthus.

5. Action of Acids : The monosaccharides are stable to the action of dilute (1.0 N) mineral acids but the concentrated acids decompose these compounds. However, aldohexoses and ketohexoses, when heated with strong mineral acids, are dehydrated and hydroxymethyl furfural is formed.



α-D-Fructopyranose 5-Hydroxymethyl furfural



α-D-Glucopyranose 5–Hydroxymethyl furfural

6. Action of Alkali : With alkali, monosaccarides react in various ways :

(a) In concentrated alkali sugar caramelizes and produces a series of decomposition products. Yellow and brown pigments develop, salts may form, many double bonds between carbon

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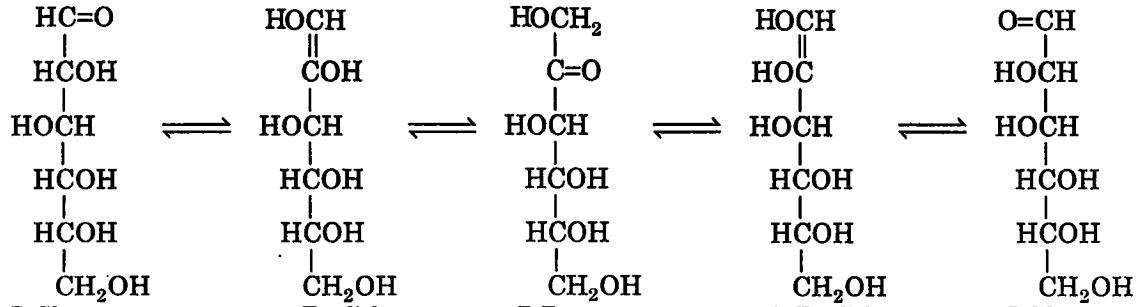
atoms are formed, and carbon-to-carbon bonds may rupture. However, the mechanism of these changes is not clearly understood.

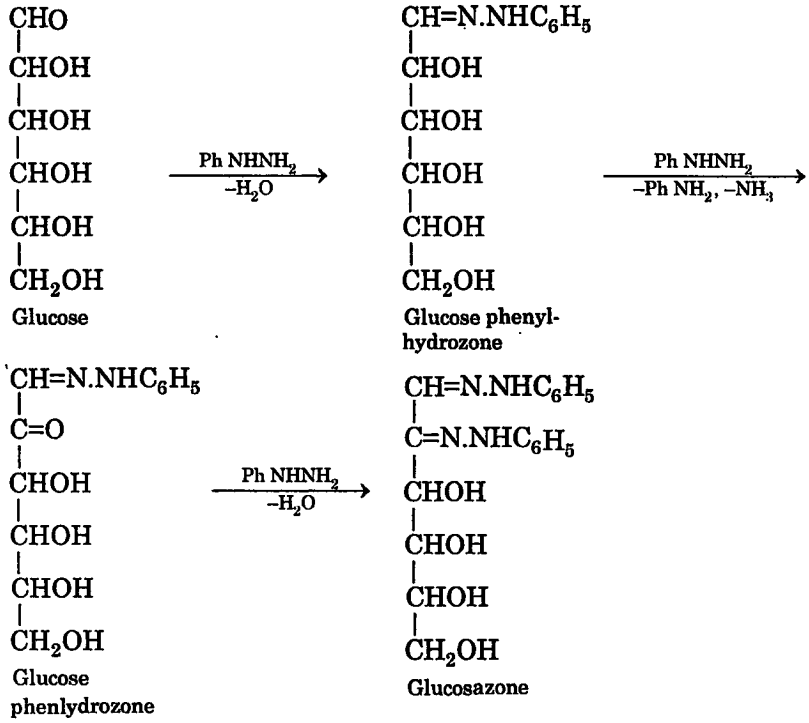
(*b*) When glucose is exposed to dilute alkali for several hours, the resulting mixture contains both fructose and mannose. If either of these sugars is treated with dilute alkali, the equilibrium mixture will contain the other sugar as well as glucose. This reaction, known as the Lobry de Bruyn—von Ekenstein transformation, is due to the enolization of these sugars in the presence of alkali. *Enediol* intermediates that are common to all three sugars are responsible for the establishment of the equilibrium. At higher concentration of alkali, the monosaccarides are generally unstable and undergo oxidation, degradation, and polymerization.



Isomerization in dilute alkali

The alkaline enolization shown has its enzymatic counterparts in that there are *isomerases* and *epimerases* that catalyze the interconversion of phosphorylated forms of these three hexoses.

7. Osazone Formation : Aldoses as well as ketoses react with hydroxylamine and phenyhydrazine. With hydroxylamine the expected product is an oxime in aldoses and ketoses. With phenylhydrazine, however, three moles of phenylhydrazine are consumed in aldoses as well as in ketoses to form *osazones*, aniline and ammonia. The overall equation may be written as follows :



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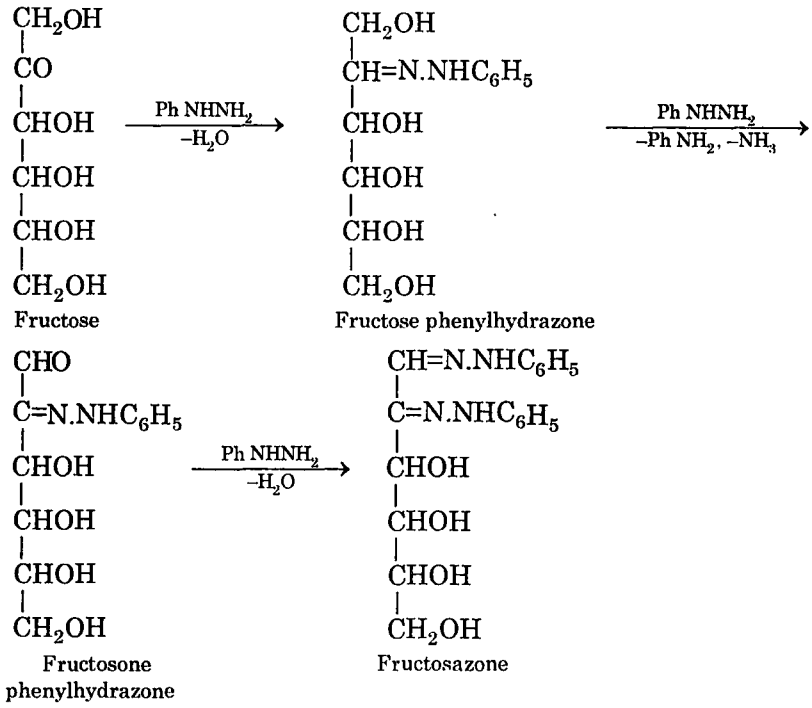
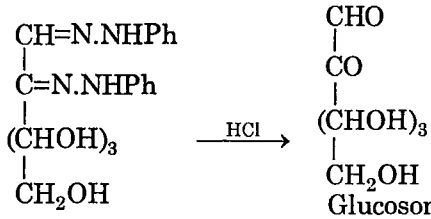
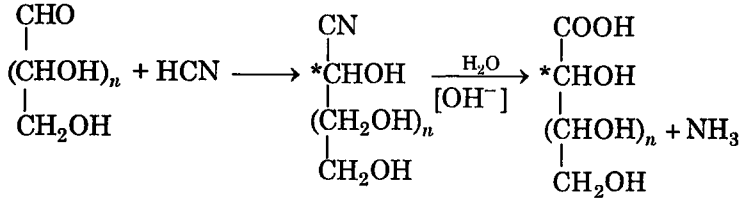


Osazones are yellow crystalline solids and are used to characterise the sugars.

When osazones are hydrolysed with hydrochloric acid, both phenylhyrazino groups are eliminated and osones are formed (osone is a dicarbonyl compound). For example, glucosazone, when hydrolysed with hydrochloric acid, yields glucosone.

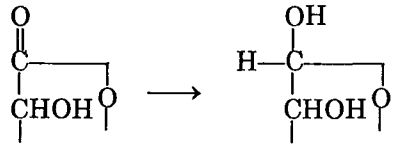


8. Addition of HCN : The general reaction for the addition of HCN to aldose may be represented as follows :



*represents the asymmetric carbon atom.

The lactone of the sugars formed in the above reaction may in turn be reduced to the corresponding hemiacetal by sodium amalgam.



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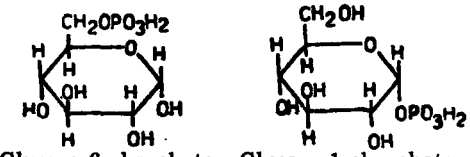
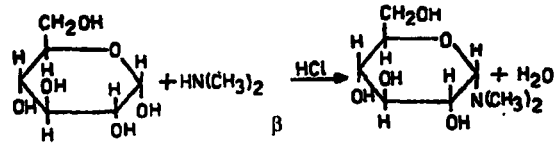
9. Phosphoric acid esters : In metabolism, phosphorylation of all sugars is the initial step in their metabolism. Thus, glucose is converted to glucose-6 phosphate. In most of the living systems, this compound is also converted into α-glucose 1-phosphate. Glucose 6-phosphate is converted into fructose 6-phosphate which gets further phosphorylated to yield 1, 6-diphosphate. The formation of these phosphoric acid esters occupies a unique formation in biochemistry. The formulae of some phosphoric acid esters are given as follows :

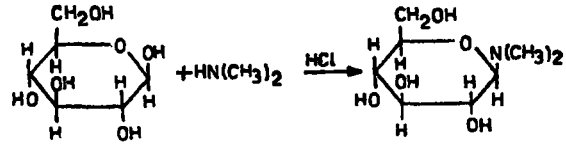


Glucose 6-phosphate Glucose 1-phosphate

10. Reaction with amines (N-glucoside formation) : When glucose is condensed with amines, Schiff's bases are not obtained but instead N-glucosides (analogous to the ordinary glucosides from alcohols) are obtained. For example, D-glucose reacts with dimethylamine amino in the presence of an acid to form two isomeric N-glucosides.

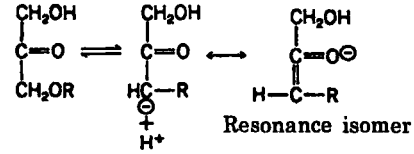


α-D-Glucose β N-Dimethyl α-D-Glucoside



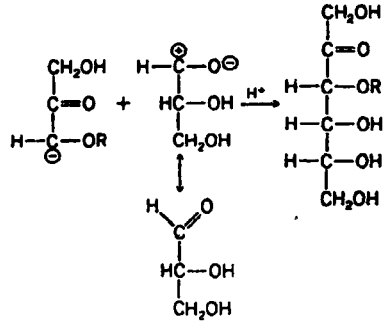
β-D-Glucose N-Dimethyl β-D-Glucoside
(N-Dimethyl β-D-Glucosylamine)

11. Aldol condensation : Another important reaction that is typical of carbohydrates and occurs frequently in biochemistry is the aldol condensation (or its reverse, the aldol cleavage). This reaction depends on the acidity of the hydrogens (α-hydrogens) on the carbon atom adjacent to a carbonyl group and the ability of the ionized ion



Resonance isomer

to be stabilized by resonance. The enolate ion is then capable of acting as a nucleophile and can attack the aldehyde group of a second sugar molecule.



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As shown above, two trioses can condense in an aldol condensation to yield a hexose. In the process, a new chiral center is produced. In theory, two hexoses with either an R or S configuration on carbon 4 would be produced. In metabolism, an enzyme assists in the formation of the enolate ion, and only one of the two possible diastereomers would be formed.

12. Fermentation : When a solution of glucose is fermented by an enzyme called *zymase*, the products are mainly ethyl alcohol and carbon dioxide.

$$\underset{\text{D-Glucose}}{C_6H_{12}O_6} \xrightarrow{\text{zymase}} \underset{\text{Ethyl alcohol}}{2C_2H_5OH} + 2CO_2$$

Similar to glucose, fructose also undergoes fermentation by the enzyme zymase present in yeast to yield mainly ethyl alcohol and carbon dioxide.

$$\underset{\text{D-Fructose}}{C_6H_{12}O_6} \xrightarrow{\text{zymase}} 2C_2H_5OH + 2CO_2$$

6.4 Structure of Monosaccharides

The simplest hexose (aldose) has a chain of carbon atoms with an aldehyde group at one end. Such a structure is optically active since it has *asymmetric carbon atoms*. Soon after the structure of glucose had been determined it was realized that the open chain formula could not account for all its properties. It could not, for example, explain the phenomenon of *mutarotation*.

Mutarotation : The usual form of glucose is the α-form. A freshly made solution of one form of glucose, α-D (+) glucose, in water has a specific rotation of +113°. When the solution is allowed to stand the rotation value falls and after 24 hours stabilizes at +52.5°. The β form of glucose β-D glucose, has a rotation of +19.7° when dissolved in water. If the solution is allowed to stand the rotation rises to give a final value of +52.5°. The phenomenon is shown by several pentoses, hexoses and reducing disaccharides and is called *mutarotation*. It is due to the conversion of the α- and β forms until an *equilibrium mixture* is reached. The value +52.5° represents the rotation of the equilibrium mixture of the two forms. These results can be interpreted in terms of *cyclic structures* of α-β-glucose.

Glucose, like all other monosaccharides, exists in two forms; the *open chain form* and the *ring form*. The open *chain form* is folded because of the tetrahedral bond angles of the carbon

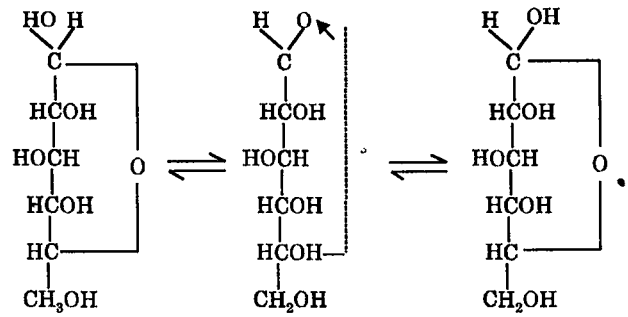

Fig. 6.7. Derivation of the ring forms of D-glucose from the open chain form.

atom. Because of this folding the ends of the molecule tend to approach each other. If the carbon atom of the aldehyde group is linked to the fifth carbon atom of the chain through an oxygen atom a *ring form* would result. Glucose exists in solutions mainly in the ring form by a formation of an intermolecular *hemi acetal*. A hemi-acetal is formed when an *aldehyde or ketone* group

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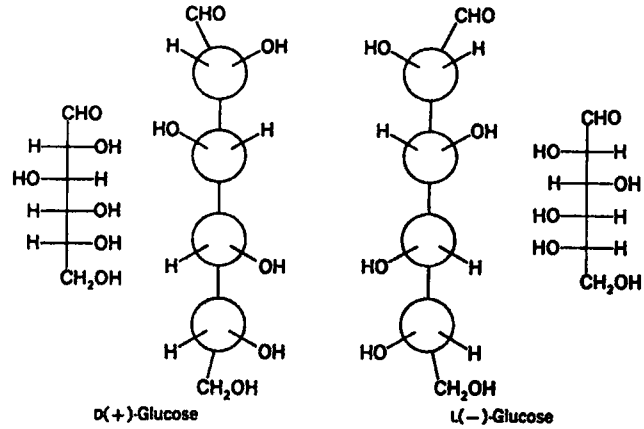
condenses with an alcoholic *hydroxyl* group within the same molecule. It will be seen that the ring form has an additional asymmetric carbon atom. The asymmetry of C-1 makes possible the two ring forms, α and β, with different optical rotations (+113° and +19.7°, respectively). The α form has the hydroxyl on the left and the β form on the right (Fig. 6.7).

Structure of Glucose

Emil Fischer received the Noble Prize in Chemistry for his studies on the structure of glucose, more specifically for establishing the configuration of the four asymmetric carbon atoms in that aldohexose relative to D(+)-glyceraldehyde. From Fischer's work, chemists were able to write the projection and ball-and-stick formulas for D- and L-glucose (Structures 6.1).

If the ball-and-stick model just represented is actually constructed and the —CHO and —CH_2OH groups are held so that they extend away from the holder (behind the plane of the paper), the remainder of the carbon atoms will tend to form a ring extending toward the holder, and the H and OH groups will project out toward the holder.

Although the aldohexoses have been considered polyhydroxy aldehydes or ketones to this point, there is abundant evidence to indicate that other forms (of glucose, *e.g.*,) exist and, indeed, predominate both in the solid phase and solution. For instance, aldohexoses undergo the Kiliani-Fischer synthesis with difficulty, although cyanohydrin formation with simple aldehydes is usually rapid. Glucose and other aldoses fail to give the Schiff test for aldehydes. Solid glucose is quite inert to oxygen, but aldehydes are notoriously autoxidizable. Finally, it is possible to prepare two crystalline forms of D-glucose. When D-glucose is dissolved in water and allowed to crystallize out by evaporation of the water, a form designated as α-D-glucose is obtained. If glucose is crystallized



Structure 6.1

from acetic acid or pyridine, another form, β-D-glucose, is obtained. These two forms of D-glucose show the phenomenon of *mutarotation*. A freshly prepared solution of α-D-glucose has a specific rotation $[\alpha]_D^{20}$ of + 113°; when the solution is left standing, it changes to +52.5°. A fresh solution of β-D-glucose, on the other hand, has an $[\alpha]_D^{20}$ of + 19°; on standing, it also changes to the same value, +52.5°.

The existence of the two forms of glucose, as well as the other anomalous properties described, was explained by the English chemist, W. N. Haworth, who showed that aldohexoses and other sugars react internally to form cyclic hemiacetals. Hemiacetal formation is a characteristic reaction between aldehydes and alcohols.

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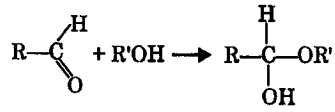

The formation of the cyclic hemiacetal structure for D-glucose is shown in Figure 6.8.

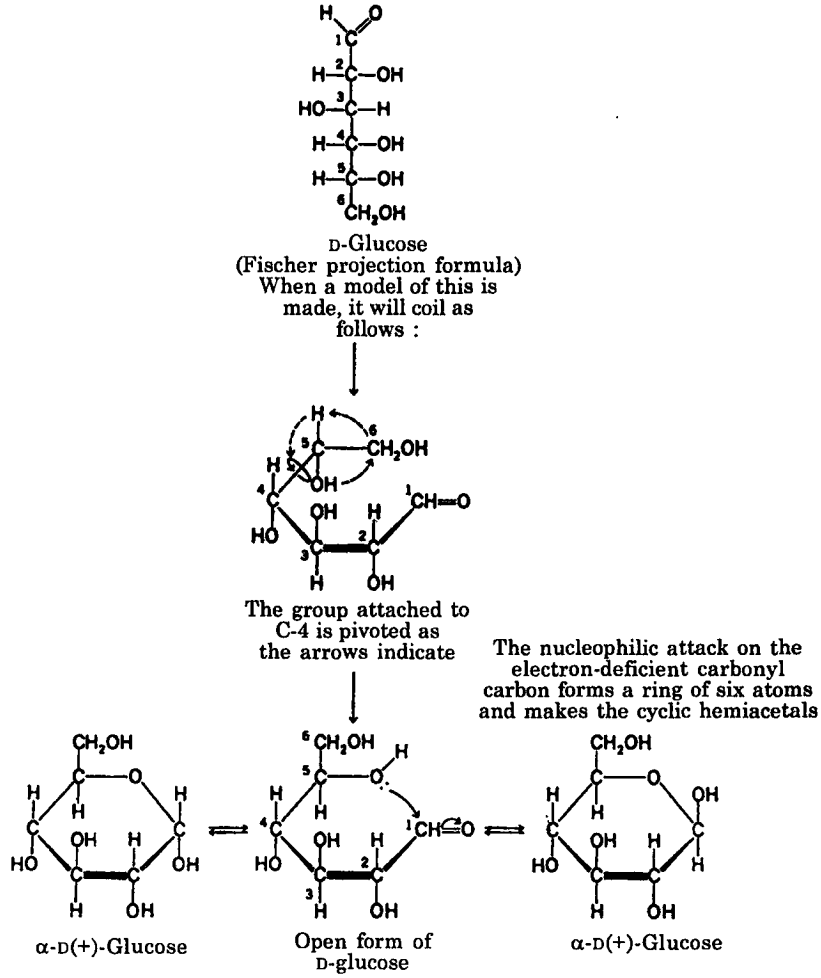

Fig. 6.8. Scheme depicting the formation of the hemiacetal forms of D-glucose. Note that an equilibrium exists between the α and β forms and the open-chain form.

Rotation of the bond between carbon atoms 4 and 5 moves the C-5 hydroxyl group into a position where it can react with the aldehyde group on C-1. This places the —CH_2OH group on C-5 above the ring. As the hemiacetal ring is formed, note that C-1 becomes a chiral center. Therefore, two diastereomeric molecules are possible. These isomers are the α and β forms of glucose; they are diastereomers, however, rather than enantiomers, for the α form differs from the β form only in the configuration at a single carbon. Since the cyclic forms of the aldohexoses have five asymmetric carbon atoms, there are 32 optical isomers of the cyclic aldohexoses consisting of 16 pairs of enantiomers.

As noted above, the angles of the tetrahedral carbon atom bend the glucose molecule into a ring. When the C-5 hydroxyl group reacts, as shown in Figure 6.8, a six-membered ring is formed.

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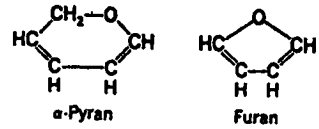
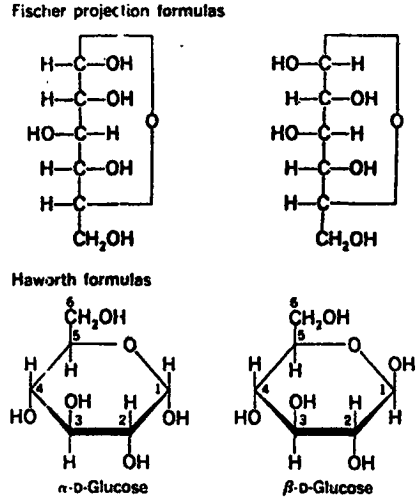
If the C-4 hydroxyl were to react, a five-membered ring would result; a seven-membered ring is too strained to permit the C-6 hydroxyl of an aldohexose to form a hemiacetal. The six-membered ring sugars may be considered derivatives of pyran, whereas the five-membered rings are considered relatives to furan.



Hence, it is customary to refer to the *pyranose* or *furanose* form of the monosaccharide. Furanose forms of the hexoses are less stable than the pyranose forms in solution; combined forms of furanose sugars (as in the fructose unit of sucrose) are found in nature, however.

Haworth proposed that these cyclic hemiacetal forms of glucose and other sugars be represented as a hexagonal ring in a plane perpendicular to the plane of the paper. The side of the hexagon that is nearer to the reader would then be indicated by a thickened line. When this is done, the substituents on the carbon atom then will extend above or below the plane of the six-membered ring. Carbon atom 6, a substituent on C-5, will therefore be above the plane of the ring. The Haworth formulas for α-D(+)-glucose and β-D(+)-glucose may then be compared with the Fischer projection formulas for these diastereomers (Structures 6.2).



Structures 6.2

With respect to assigning structures to the α- and β-anomers, Fischer originally suggested that, in the D-series, the more dextrorotatory compound be called the α-anomer whereas, in the L series, the α-anomer would be the more levorotatory substance. Later, Freudenberg proposed that the α- and β-anomers be classified with respect to their configuration rather than sign or magnitude of rotation. The relationship of the anomeric hydroxyl to the reference carbon atom is easy to see when Fischer projection formulas for the ring structures are used. In these projections, the α-anomer is the isomer in which the anomeric hydroxyl is on the same side (*cis*) of the carbon chain as the hydroxyl group on the reference carbon atom. If the reference hydroxyl group happens, as it does in α-D-glucopyranose, to be involved in ring formation, then the anomeric hydroxyl of the α-isomer is on the same side as the ring structure formed by the oxygen bridge. In the β-anomer, the hemiacetal hydroxyl group is *trans* to the hydroxyl on the reference carbon atom.

The assignment of configuration to the anomeric carbon atom is less readily seen with the Haworth formulas. In the case of D-hexoses and D-pentoses, the α-anomer has the anomeric

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hydroxyl written below the plane of the ring. The β-anomer then has the anomeric hydroxyl above the plane of the ring. Examples are given in Figure 6.9.

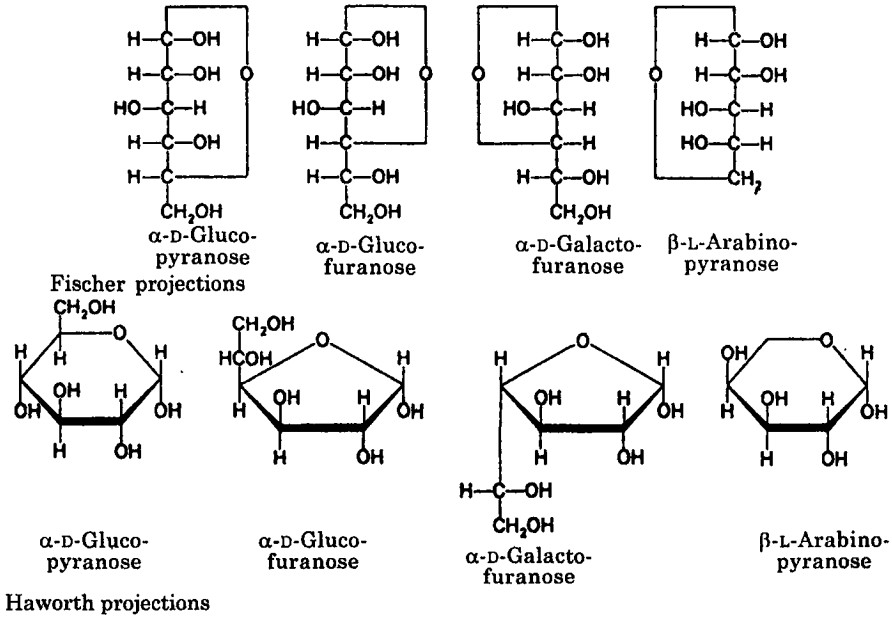
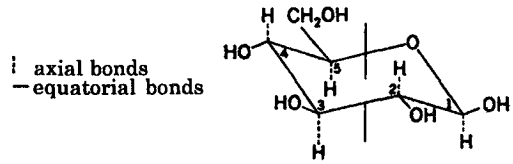


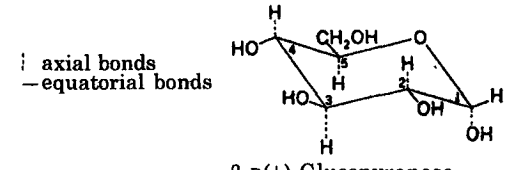
Fig. 6.9. Fischer and Haworth formulas of some common monosaccharides.

There is still one final aspect of the structure of glucose to be mentioned; this is its *conformation*. Because the C—O—C bond angle of the hemiacetal ring (111°) is similar to that of the C—C—C ring angles (109°) in cyclohexane, the pyranose ring of glucose, rather than forming a true plane, is puckered in much the same way as cyclohexane. Like cyclohexane, glucopyranose can exist in two conformations : the *chair* and *boat* forms. The chair conformation



of glucose minimizes torsional strain and further, the conformational structure in which a maximum number of bulky groups (—OH and —CH_2OH) are *equatorial* rather than *axial* to an axis passing through the ring is preferred.

The following diagram shows that β-D(+)-glucopyranose can achieve a conformation in which all bulky groups are equatorial (or perpendicular) to an axis passing through the plane of the ring. This conformation is thermodynamically more stable than that in which the hydroxyls and the —CH_2OH are axial (parallel to the axis shown). α-D-Glucopyranose can have a conformation in which all bulky groups *except* the anomeric hydroxyl are equatorial, and the preferred structure for this form may be represented as



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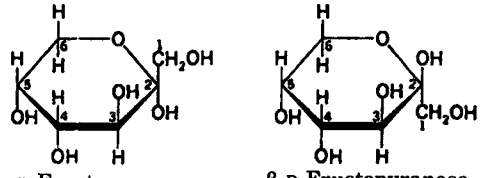
Therefore, one of the two anomers, namely the β-anomer with *all* bulky groups equatorial, should predominate in a solution over the α-isomer with one axial group, the anomeric hydroxyl. Thus, in aqueous solution, β-D(+)-glucopyranose is present to the extent of about 63% after mutarotation, whereas α-D(+)-glucopyranose comprises about 36%. The linear polyhydroxy aldehyde form accounts for less than 1% of the total carbon present as glucose (see Figure 6.8).

6.5 Structures of Other Monosaccharides

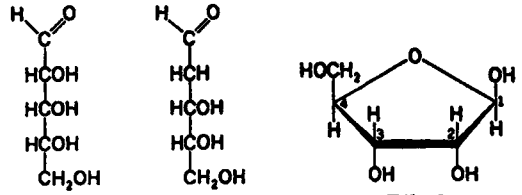
Pyranose forms for the other aldohexoses may be written by the proper arrangement of the hydroxyl groups on C-2, C-3, and C-4. Similarly, the Haworth formulas for α-D-fructopyranose and β-D-fructopyranose may be written as shown in Structure 6.3. Note, however, that the five-member furanose structure is the one encountered for fructose when the hemiketal (from the ketone group of the ketohexoses) group is substituted as in sucrose and fructosans.



α-D-Fructopyranose β-D-Fructopyranose

Structures 6.3

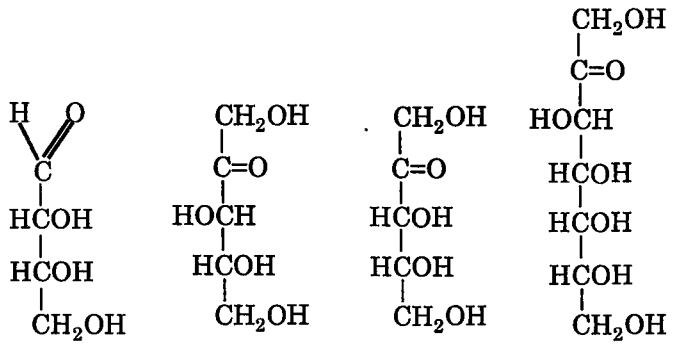
The ubiquitous pentose, D-ribose, a component of ribonucleic acid, exists as a furanose; 2-deoxy-D-ribose, a component of 2-deoxyribonucleic acid, is also a furanose sugar. Both α-and β-isomers can exist in solution, but the β-isomer is the one that is found in the nucleic acids.



D-Ribose 2-Deoxy-D-ribose β-D-Ribofuranose

Structures 6.4

Four other monosaccharides that play important roles in the metabolism of carbohydrates during photosynthesis are the aldotetrose, D-erythrose; the keto-pentoses, D-xylulose and D-ribulose; and the ketoheptose, D-sedoheptulose.



D-Erythrose D-Xylulose D-Ribulose D-Sedoheptulose

Although five-membered hemiacetal (erythrose) or hemiketal (xylulose, ribulose) structures of these monosaccharides may be written, the metabolically active forms are the phosphate esters in which the primary alcohol (—CH_2OH) group has been esterified with H_3PO_4, thereby preventing its participation in a ring structure.

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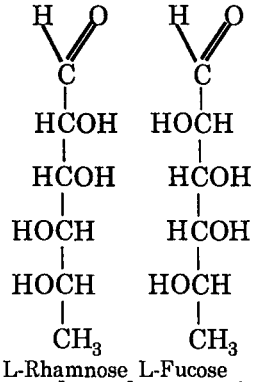
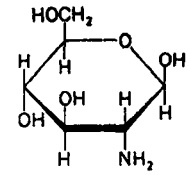
Two other deoxy sugars are found in nature as components of cell walls. Those are L-rhamnose (6-deoxy-L-mannose) and L-fucose (6-deoxy-L-galactose).

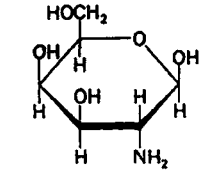


L-Rhamnose L-Fucose

Two amino sugars, D-glucosamine and D-galactosamine, exist in which the hydroxyl group at C-2 is replaced by an amino group. The former is a major component of chitin, a structural polysaccharide found in insects and crustaceans. D-Galactosamine is a major component of the polysaccharide of cartilage. Their hemiacetal forms are shown here.



2-Deoxy-2-amino-β-D-glucopyranose
(β-D-Glucosamine)



2-Deoxy-2-amino-β-D-galactopyranose
(β-D-Galactosamine)

6.6 Some Important Monosaccharides

1. Trioses : These contain three carbon atoms, *e.g., glyceraldehyde* (an *aldose*) and *dihydroxyacetone* (*a ketose*) (Fig. 6.10).

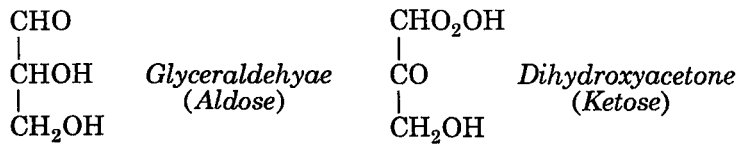
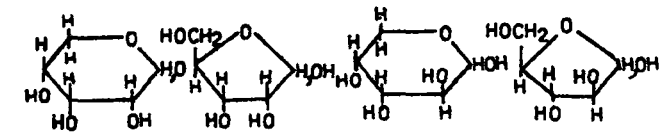


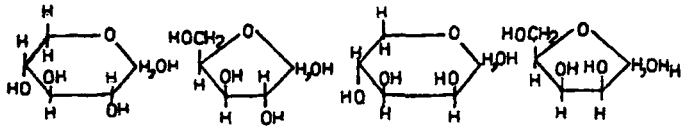
Fig. 6.10. Trioses (Glyceraldehyde (aldose) and dihyroxyacetone (ketose).

2. Tetroses : These contain four carbon atoms in the chain. Only two are possible, *erythrose* and *threose*. On nitric acid oxidation erythrose yields *mesotartaric acid* while threose gives *D*(–) *tartaric acid.*

3. Pentoses : These contain five carbon atoms in the molecule (Fig. 6.11). The important pentoses are :



Ribose pyranose form | Ribose furanose form | Arabinose pyranose form | Arabinose furanose form



Xylose pyranose form | Xylose furanose form | Lyxose pyranose form | Lyxose furanose form

Fig. 6.11. Pentoses.

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Ribose—found in ribonucleotides and in RNA (Fig. 6.12). Also present in the coenzymes ATP, FAD, NAD and NADP.

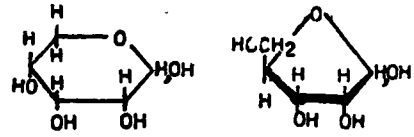

Fig. 6.12. D-Ribose.

Dexoyribose—found in deoxyribonucleotides and DNA.

D-Arabinose—Occurs in glycosides of tuberculosis bacilli.

Ribulose—an important compound in photosynthesis.

Pentoses are also present as pentosans in wood gums.

4. Hexoses : These are 6-carbon atom sugars. Hexoses of biological importance are *glucose, galactose, mannose* and *fructose* (Fig. 6.13).

D-glucose is the most widely distributed sugar. It is sweet tasting, like all sugars. Oxidation of glucose provides the immediate energy requirement of the cell. Carbohydrates are transported in the blood in the form of glucose. The polysaccharide *glycogen* is built up of glucose units. It is found in fruit juices.

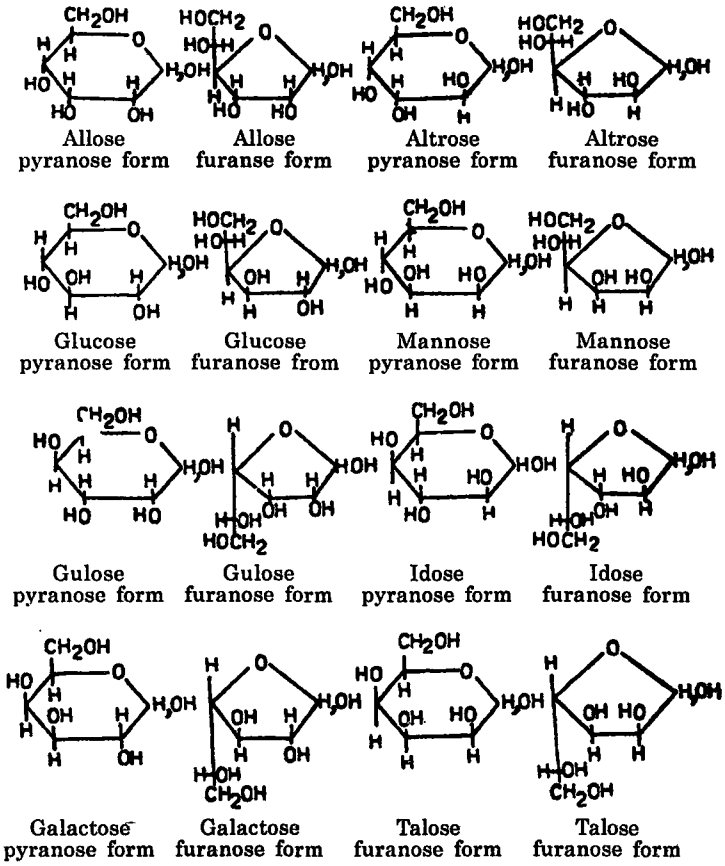

Fig. 6.13. Hexoses.

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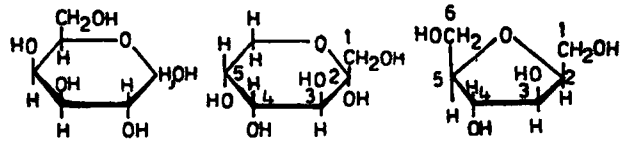
D-Galactose is found in glycolipids of nervous tissue. It is also a component of the disaccharide *lactose*. The main function of galactose appears to be structural (Fig. 6.14).



D-Galactose | α-D-Fructose pyranose form | α-D-Fructose furanose form

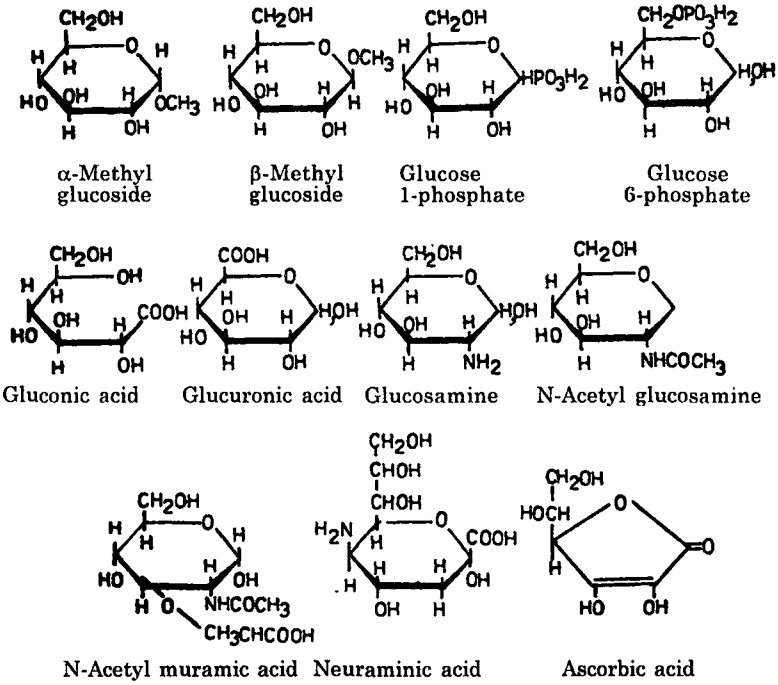
Fig. 6.14. D-galactose and D-fructose.

D-Mannose is widely distributed as *monnans* in plants. Small amounts are present in some glycoproteins. Mannose is converted to glucose in animals.

D-Fructose is the sweets of sugars. It is found in fruit juices, honey and seminal fluid. In the free state it is present in the *pyranose* ring form, and when combined, in the *furanose* form.

6.7 Some Derived Monosaccharides

Derived monosaccharides differ from *normal monosaccharides with respect of aldoses* and *ketoses*. They include the *glycosides, sugar, phosphates, gluconic acid, glucuronic acid, amino sugars* and *vitamin C* (Fig. 6.15).



α-Methyl glucoside | β-Methyl glucoside | Glucose 1-phosphate | Glucose 6-phosphate

Gluconic acid | Glucuronic acid | Glucosamine | N-Acetyl glucosamine

N-Acetyl muramic acid | Neuraminic acid | Ascorbic acid

Fig. 6.15. Derived monosaccharides

1. Glycosides : The hydroxyl group on carbon 1 in the sugar molecule can be replaced by other radicals, forming compounds known as *glycosides*. Thus if a *methyl group* replaces the *hydroxyl group* of glucose it results in the formation of *methyl glycosides*. The are two methyl glycosides possible, α and β, corresponding to the α and β forms of glucose. Glycosides formed

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from *galactose* are known as *galactosides*. Among the complex glycosides occurring in nature are *digoxin*, which acts on the heart, and the antibiotic *streptomycin*.

2. Sugar phosphates : C-1 or C-6 of glucose can react with *phosphoric acid* (H_3PO_4) to yield *glucose-1-phosphate* and *glucose 6-phosphate*, respectively. These compounds play an important part in carbohydrate metabolism.

3. Gluconic acid and glucuronic acid : Oxidation of C-1 of the glucose molecule to a *carboxyl group* yields *gluconic acid*. Production of a carboxyl group at C-6 yields *glucuronic acid*. The corresponding terms for any hexoses are *hexonic acid* and *hexuronic acid*.

Glucuronic acid is an important constituent of complex polysaccharides and is also an important coupling agent. Many drugs, pesticides, and environmental pollutants and hormones are coupled with glucoronic acid and excreted in urine or bile as *glucuronides*.

4. Amino sugars or hexosamines are formed when an amino sugar is introduced into hexoses. Thus glucose with NH_2 at C-2 forms *glucosamine*. This compound is extensively found in complex polysaccharides, usually in the form of its acetyl derivative *N-acetyl glucosamine*. *N-Acetyl galactosamine*, the amino derivative of galactose, is found in *glycoproteins* and *glycolipids*. *N acetyl muramic acid* is found in the polysaccharides of the cell wall of some bacteria. *Neuraminic acid* gives acetyl derivatives called *sialic acids*. These are constituents of some *glycoproteins* in bone and connective tissue and *glycolipids* of the nervous system.

5. Vitamin C (Ascorbic acid) is a sugar acid of biological importance.

6.8 Oligosaccharides

Oligosaccharides are short chains of monosaccharides linked together by glycosidic bonds. In the case of oligosaccharides linked to proteins (glycoproteins) or lipids (glycolipids), the oligosaccharide is not a repeating unit but consists of a range of different monosaccharides joined by a variety of types of bonds. In glycoproteins, two main types of oligosaccharide linkages exist :

1. *O-linked oligosaccharides* attached to the protein *via O-glycosidic bonds* to the OH groups of serine or threonine side-chains.
2. *N-linked oligosaccharides* attached to the protein *via N-glycosidic bonds* to the NH_2 groups of asparagine side-chains (Fig. 6.16). All N-linked oligosaccharides have a common pentasaccharide core of two GlcNAc and three Man residues but the nature of the side-chains differs (Fig. 6.16). In the *high mannose type* of N-linked oligosaccharide, typically two to six additional Man residues are joined to the pentasaccharide core [*e.g.*, Fig. 6.16 (*a*)]. The *complex type* of N-linked oligosaccharide contain two to five outer branches attached to the Man of the polysaccharide core; these branches contain different combinations of GlcNAc, Gal, sialic acid (*N*-acetylneuraminic acid), mannose and L-fucose. Fig. 6.16 (*b*) shows a complex oligosaccharide with two outer branches.

Glycosidic linkage : A sugar molecule can combine with an identical or a different type of a sugar molecule. The linkage between two monosaccharide sugar molecules is called a *glycosidic linkage* or *glycosidic bond*.

A sugar molecule has several reactive hydroxyl groups. The hydroxyl group of number one carbon atom (C-1) is known as the *glycosidic hydroxyl*. It is very reactive and readily forms a glycosidic link with a hydroxyl group of another sugar molecule. When such a linkage occurs, one hydrogen atom and one hydroxyl group are eliminated to form H_2O. This process is one of

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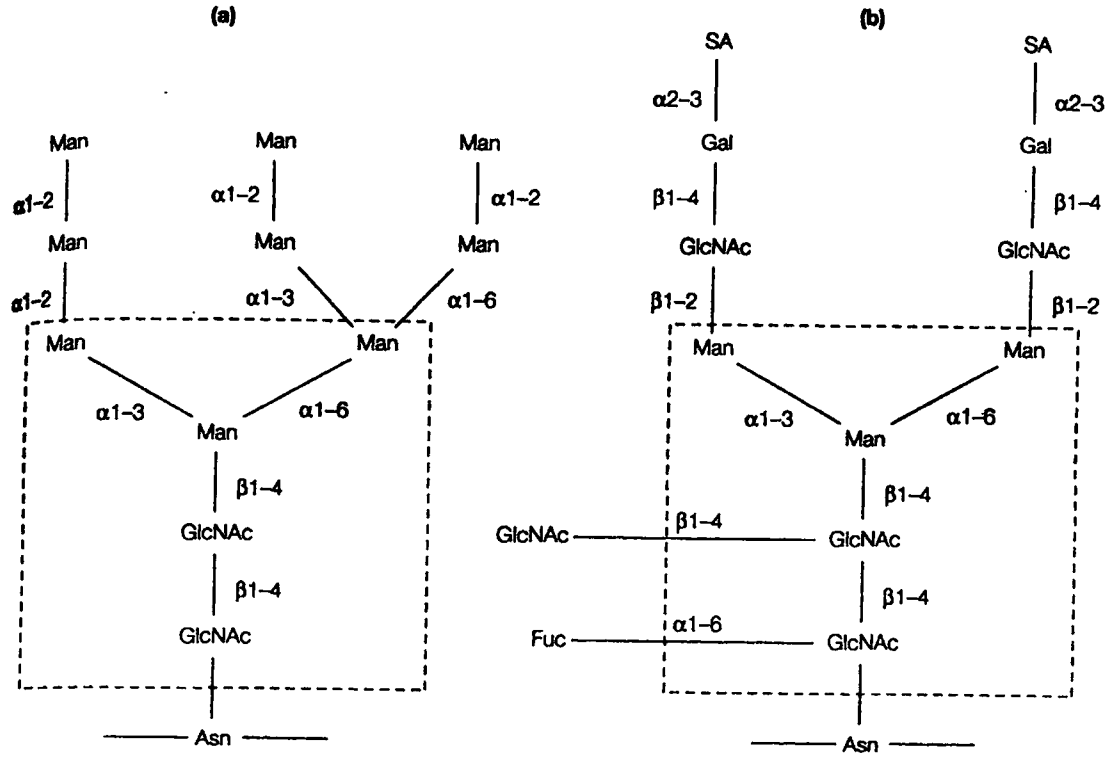

Fig. 6.16. Examples of (*a*) high mannose type and (*b*) complex type oligosaccharides. In each case, the sugars that comprise the common pentasaccharide core are boxed. SA, sialic acid.

condensation and is in fact *dehydration synthesis*. The reverse process in which the molecule is cleaved with the incorporation of the elements of water is called *hydrolysis*.

A linkage between C-1 of one monosaccharide unit and C-4 of another is called the 1, 4 *linkage*. Such linkage occurs in disaccharides and in the unbranched chains of polysaccharides. The 1, 4 linkage between two hydroxyl groups in the α position is called the α-1, 4 *linkage* (*e.g., maltose*). Similarly a linkage between two β hydroxyl groups is called the β-1-4 *linkage* (*e.g.*, in *lactose*). Linkage can also take place between C-1 of one monosaccharide unit and C-6 of another. Such a linkage occurs at the point of branching of a chain and is known as a *1, 6 linkage* (*e.g.*, in *isomaltose*).

The α or β configurations can be determined by specific enzymes. The enzyme *maltase* attacks only α-glycosides, while *emulsin* hydrolyses only β glycosides. *Starch* has α-glycosidic bonds while *cellulose* has β-glycosidic bonds. The α bonds of starch can be cleaved by enzymes which do not cleave the β bond of cellulose. This explains why starch is a valuable food while cellulose has no food value for most animals.

Maltose and *lactose* both have unlinked potential aldehyde and are therefore reducing sugars. As long as the bond is not there between two C-1 atoms, a free aldehyde or ketone group is left on the disaccharide. The disaccharide therefore shows all the reactions associated with the groups. In *sucrose* linkage is through both the potentially reducing groups of the monosaccarides. Sucrose is therefore non-reducing.

Oligosaccharides are composed of 2 to 9 monosaccharide units or their derivatives. This commonly employed definition of oligosaccharides is an arbitrary one. In general it can be said

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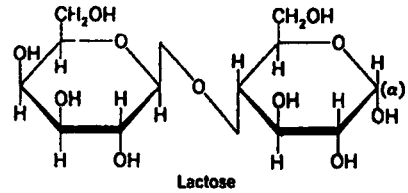
that oligosaccharides contain fewer sugar residues than polysaccharides. The oligosaccharides are named according to the number of monosaccharide units they contain. Thus, *disaccharides* contain two monosaccharide units, *trisaccharides* three, *tetrasaccharides* four, *pentasaccharides* five, etc.

Disaccharides : These are the most important of oligosaccharides. They occur as constituents of both plant and animal cells. A disaccharide is formed by condensation of two monomers of monosaccharides with elimination of one molecule of water. The important disaccharides are *lactose, maltose and sucrose.*

Lactose or milk sugar : It occurs in the milk of mammals and is synthesized in the mammary glands. It may also be present in the urine during pregnancy. Lactose is made up of one *glucose* and one *galactose* unit linked by *β-1 → 4 linkage.* Souring of milk takes place when bacteria found in milk convert *lactose* into *lactic acid.*

Lactose is a disaccharide found in milk; on hydrolysis, it yields one mole each of D-galactose and D-glucose. It possesses a β(1 → 4) linkage, is a reducing sugar, and can undergo mutarotation, α-Lactose has the following formula in which the configuration at the reducing end of the disaccharide is shown as α.

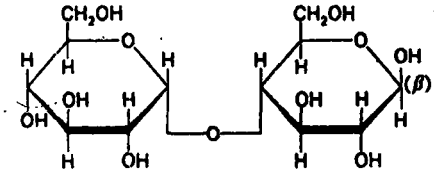


Lactose

Being the major carbohydrate in milk, lactose is extremely important in the nutrition of young mammals. Most of the world's human population relies on this sugar as a major form of energy during the first years of life. Lactose itself cannot be absorbed into the blood, but must first be hydrolyzed to its constituent monosaccharides by intestinal lactose. This enzyme is abundant in nursing infants but tends to disappear with age. Only Northern Europeans and a few other African peoples retain the enzyme in adulthood. Most other human groups have little intestinal lactose as adults and some, especially Mediterranean peoples and Orientals, may exhibit an intolerance to the sugar. In these people, high dietary intake of lactose results in intestinal disturbance in the form of diarrhea and pain.

Maltose or malt sugar : It is found in germinating seeds and malt. It is also produced during the digestion of starch by *α-amylase.* Maltose on hydrolysis yields two glucose molecules. This shows that it is made up of two glucose units. The hydroxyl on C-1 of one glucose molecule is linked to the hydroxyl group on C-4 of the second glucose molecule through an α-glycosidic linkage. Such a linkage is known as an *α–1, 4 glycosidic linkage.*

Among the disaccharides encountered is the sugar maltose; this sugar is obtained as an intermediate in the hydrolysis of starch by enzymes known as amylases. In maltose, one molecule of glucose is linked through the hydroxyl group on the C-1 carbon atom in a glycosidic bond to the hydroxyl group on the C-4 of a second molecule of glucose.



Maltose

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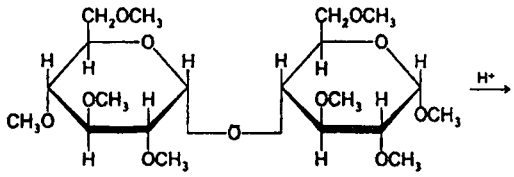
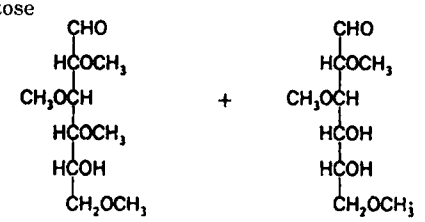
The glycosidic linkage between the two glucose residues is designated as α(1 → 4) to specify that the anomeric carbon involved in the glycosidic bond has the α-configuration and that it is linked to the 4-position of the second glucose molecule. This second glucose moiety possesses a free anomeric hydroxyl that can exist in either the α- or β-configuration (the β-isomer is shown); this free anomeric hydroxyl thus confers the property of mutarotation on maltose, and the disaccharide is a reducing sugar. That maltose has the structure shown was determined by analyzing the two products obtained on acid hydrolysis of its *octa* methyl derivative. The fully methylated maltose yields 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,6-tri-*O*-methyl-D-glucose on hydrolysis. While the anomeric carbon of maltose is methylated on treatment of the disaccharide with dimethyl sulfate, this *O*-methyl glycosidic bond as well as the glycosidic bond between the two glucose units of the disaccharide is acid labile charide is acid labile and both are cleaved on hydrolysis with acid.

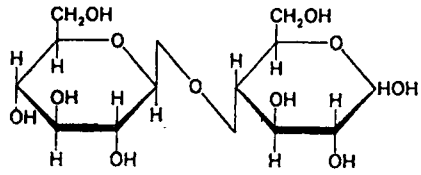


Octamethyl-D-maltose



2,3,4,6-Tetra-O-methyl-D-glucose 2,3,6-Tetra-O-methyl-D-glucose

Cellobiose : The disaccharide cellobiose is identical with maltose except that the former compound has a β(1 → 4) glycosidic linkage. Cellobiose is a disaccharide formed during the acid hydrolysis of cellulose. It is a reducing sugar and undergoes mutarotation. Treatment of cellobiose with dimethyl sulfate would also yield an octamethylated sugar, and acid hydrolysis would yield the same products that were obtained from octamethyl maltose.



Cellobiose

Isomaltose : It is also formed by combination of two glucose units, but it is 1 → 6 link. It means that C-1 of one unit is connected through oxygen bridge to C-6 of other unit. It exists as a unit in glycogen, amylopectin and some bacterial dextrans. It is hydrolysed by the enzyme, oligo-1, 6-glucosidase in the intestines producing two glucose units. The full name is 6-O-α-D-glucopyranosyl D-glucopyranose. C-1 of one unit being free, it has reducing property.

Isomaltose, another disaccharide obtained during the hydrolysis of certain polysaccharides, is similar to maltose except that it has an α(1 → 6) linkage. Exhaustive methylation and acid hydrolysis of octamethyl isomaltose would yield 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,4-tri-*O*-methyl-D-glucose.

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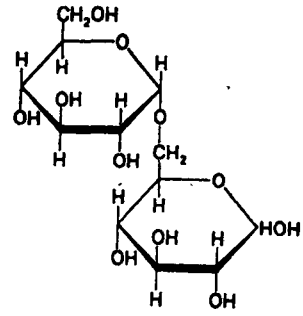
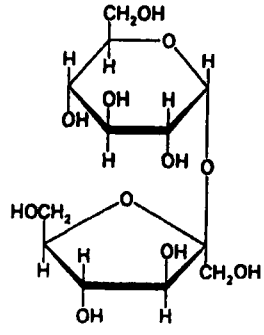


Isomaltose

Sucrose : Sucrose, the sugar of commerce, is produced by higher plants; sugar beets and sugar cane are major commercial sources. On hydrolysis, sucrose yields one molecule each of glucose and fructose, but, in contrast to all the other mono- and disaccharides described previously, sucrose is not a reducing sugar. This means that the reducing groups in both of the monosaccharide components must be involved in the glycosidic linkage between the two sugars. That is, the C-1 and C-2 carbon atoms, respectively, of the glucose and fructose moieties must be covalently linked in a glycoside bond. Permethylation studies of sucrose has shown that it must have the following structure with an α-configuration on the glucose subunit and a β-configuration on the fructose moiety.



Sucrose

Sucrose is a major product of plant photosynthesis. Sucrose is also the form in which carbohydrate, produced in the leaves by photosynthesis, is transported into storage organs such as developing seeds, tubers, or roots. It has been suggested that sucrose has an advantage, both as a storage product and a transport form of carbohydrate, over glucose and the other common sugars since both of its anomeric carbon atoms are protected from oxidative attack.

Sucrose, and maltose to a lesser extent, are important carbohydrate components of the human diet. However, they cannot be directly absorbed into the body and, like lactose, must first be hydrolyzed by specific enzymes, sucrose and maltase, found in the intestinal mucosa. Sucrose is used extensively as a sweetening agent in the food industry. It is readily available and is sweeter than the other common sugars maltose, lactose, and glucose. Only fructose is sweeter, and today enzymically produced mixtures of glucose and fructose, obtained from corn and other plant starches, are replacing sucrose as a commercial sweetener. Such mixtures are nutritionally equivalent to sucrose on a weight basis and significantly sweeter.

Fructose exists in the furnanose form when combined in sucrose. C-1 of glucose and C-2 of fructose participate in glycoside formation. Configuration in glucose is α while in fructose it is β.

Trehalose, which is found in insects and fungi, is another non-reducing disaccharide.

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Table 6.2
MONOSACCHARIDE UNITS AND GLYCOSIDIC LINKAGES OF SOME DISACCHARIDES

Disaccharide	*Monosaccharide constituents*	*Glycosidic linkage*
Lactose	D-glucose + D-galactose	β-1,4
Maltose	D-glucose + D-glucose	α-1,4
Cellobiose	D-glucose + D-glucose	β-1,4
Isomaltose	D-glucose + D-glucose	α-1,6
Sucrose	D-glucose + D-fructose	α-1, β 2

Trisaccharides : These have three monosaccharide units and the general formula $C_{18}H_{32}O_{16}$. *Raffinose* consists of three monosaccharide units D-glucose, D-fructose and D-galactose. Among the other trisaccharides are *mannotririose, rabinose, rhamminose, gentianose* and *melezitose.*

6.9 Polysaccharides

The term polysaccharide is usually employed for polymers containing at least 10 monosaccharide units. Polysaccharides are named after their monosaccharide monomers by changing the *—ose* ending of the monosaccharide to *-an.* Thus *cellulose,* a polysaccharide formed of *glucose* units, is called *glucan. Fructose* polymers are called *fructans* (previously *fructosans*) and *pentose* polymers *pentans* (formerly *pentosans*). Since the term *glycose* is used for any monosaccharide, the general name for any polysaccharide is *glycan.* If a polysaccharide contains more than one type of sugar unit the two sugars are named in alphabetical order. Thus, a polysaccharide consisting of *D-glucose* and *D-mannose* units is termed *D-gluco-D-mannoglycon.* Some old names which have not been changed to conform with the—an ending include *starch, cellulose, pectin, amylopectin, inulin, chitin* and *heparin.*

Polysaccharides have been classified as *homopolysaccharides (homoglycans)* and *heteropolysaccharides (heteroglycans). Homoglycans* contain only one type of monosaccharide while *heteroglycans* contain at least two types. Most polysaccharides contain one or two types of glucose units. Polysaccharides containing three types of glucose units are less common. The term homoglycan is used when at least 95% of the sugars are of one type, because it is very rarely that a polysaccharide can be obtained in pure form.

Polysaccharides are formed by condensation of many molecules of monosaccharides with corresponding elimination of water molecules. The monosaccharide units are joined by *glycosidic bonds.*

A. Homopolysaccharides (homoglycans) : Some of the better known homoglycans are :

Glucans (of *glucose* monomers)—starch, glycogen, cellulose, chitin.

Galactans (of *galactose* monomers)—agar, pectin, galactan from snails.

Mannans (of *mannose* monomers)—yeast mannan.

Xylans (of *xylose* monomers)—hemicellulose xylan.

Fructans (of *fructose* monomers)—inulin.

Starch : Starch is the reserve substance in plant cells. It is a polymer of D-*glucopyranose* units linked by α-1, 4. It consists of a mixture of *amylose* and *amylopectin* in the proportion of

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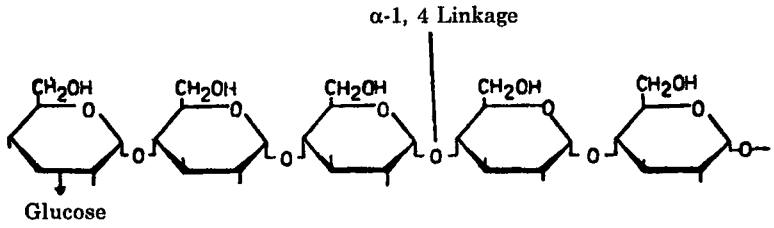
1 to 4. Both are high molecular weight compounds. *Amylose is linear* while *amylopectin* is *branched.* Treating starch with hot water dissolves amylose, while amylopectin remains.

Amylose : It is the water soluble portion of starch grains, often termed *soluble starch.* It gives intense blue colour with iodine with which it forms a complex. Hydrolysis produces maltose units, and finally glucose only. Molecular weight varies from 4000 to 400,000. The structure is believed to be an unbranched chain of D-glucose units with 1 → 4 linkages. There are both α-amylose and β-amylose, producing on hydrolysis, α-glucose and β-glucose, respectively. Structure of α-amylose is given in Fig. 6.17.

(*a*)



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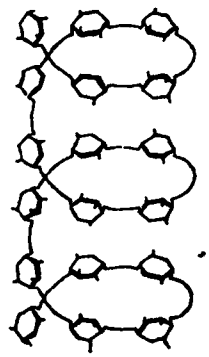

Fig. 6.17. (*a*) Structure of amylose showing a- 1,4 linkage; (*b*) Helical coil of amylose on suspension in water.

Amylopectin (Fig. 6.18) has over 1,000 glucose residues and molecular weight is about 2,00,000 to 1,000,0000. It has a branched structure. Amylopectin consists of glucose units linked by α-1, 4 glycosidic bonds. There are occasional α-1.6 glycosidic bonds which cause branching.

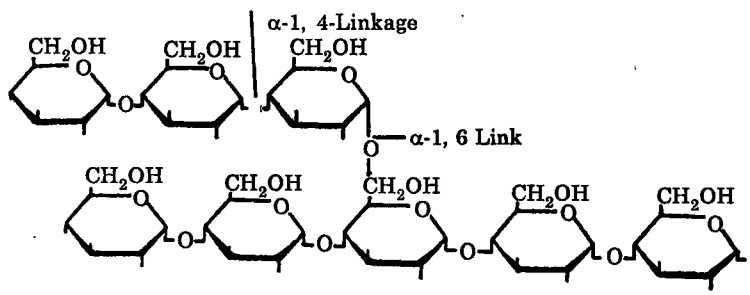

Fig. 6.18. Amylopection (branched polysaccharide).

Dextrins : Starch on hydrolysis yields lower molecular weight polysaccharides, and finally glucose or maltose. Partial hydrolysis results in substances called *dextrins.* At first *erythrodextrins* are formed which give red colour with iodine. Further hydrolysis results in the formation of

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achroodextrins which do not give the red colour with iodine. Finally reducing sugars appear. Dextrins are easily digested and are therefore used for feeding infants. When mixed with milk they prevent formation of curds in the stomach. The enzymes which bring about hydrolysis of starch are called *amylases.*

Glycogen (Fig. 6.19) : It is the major reserve carbohydrate in animals and is therefore also called *animal starch.* It is found mainly in the *liver* and in *muscles.* The glycogen in the *liver* supplies glucose to all tissues through the blood. *Muscle glycogen* on the other hand is available during contraction of muscle.

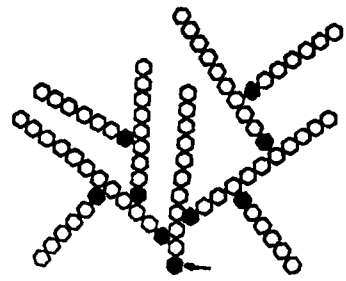
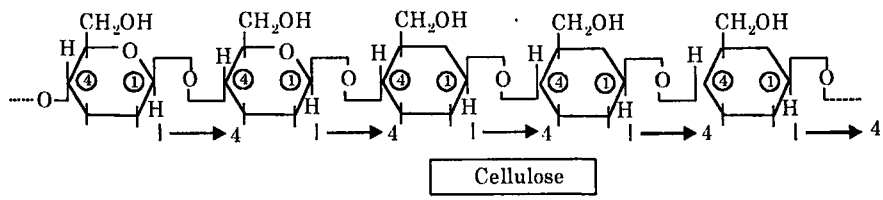


Fig. 6.19. Structure of glycogen molecule.

Chemically glycogen is analogous to starch. Both are glucans consisting of glucose units linked by α-1,4 glycosidic bonds, with branch chains formed at α-1, 6 bonds. They differ mainly in their molecular weights and degree of branching. Molecular weights range from about 3,00,000 to 100 million. The branching chain of glycogen average about 12 glucose units as compared with the 25 or so for starch. Glycogen has branch points about every 8-10 glucose units. About 5,000 to 15,000 glucose units make up glycogen. Glycogen is non-reducing and gives a red colour with iodine.

Cellulose : It is the most important structural component of the cell wall of plants. It is also found in a few microorganisms and lower organisms. It is not digested by the digestive enzymes of man, and therefore forms the 'bulk' or 'roughage' of food. In contrast to starch and glycogen, cellulose is insoluble in ordinary solvents and is not hydrolysed by boiling dilute acids. It gives no colour with iodine.

Cellulose is a linear polymer of β-D glucose units connected through β-1, 4 glycosidic bonds. The linear chains form *microfibrillae* or bundles of parallel chains held together by hydrogen bonds. This micellar structure gives mechanical strength to cellulose. In contrast to starch, the β-1,4 linkages of cellulose are highly resistant to acid hydrolysis. Partial hydrolysis yields *cellotetrose, cellotriose,* and *cellobiose* consisting of four, three and two monosaccharide units, respectively. Strong mineral acid is required for complete hydrolysis by *glycosidases* found in the digestive tract of humans and higher animals. This accounts for the resistance of cellulose to digestion. Some snails, however, produce an enzyme (*cellulase*) that hydrolyses cellulose, and termites also contain a similar enzyme. Bacteria found in the rumen of cattle and other ruminants can also hydrolyse cellulose, and termites also contain a similar enzyme. Bacteria found in the rumen of cattle and other ruminants can also hydrolyse cellulose. The D-glucose produced can be further metabolized by the bacteria.



Chitin : It is an important polysaccharide of invertebrates. It is found in the hard exoskeleton of insects and crustaceans and in the cell walls of fungi. It is polyglycan consisting *N-acetyl D-glucosamine* units connected through β-1,4 glycosidic linkages. Like cellulose it consists of parallel chains of molecules held together in boundles by hydrogen bonds.

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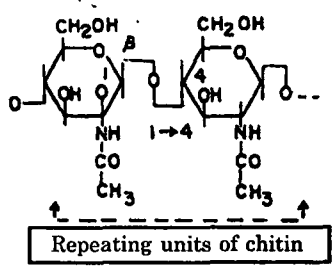

Agar is a galactan consisting of both D and L galactose. It is used as a bacteriological culture medium. It is a vegetable mucilege obtained from sea weeds and is the sulphuric acid ester of a complex polysaccharide of galactose, usually combined with K^+, Na^+, Ca^{2+}, etc. It dissolves in not water, forming a solution which, on cooling, sets to a gel. Agar gels are very valuable as a supporting agent in bacterial culture media. Both D- and L-galactose units are present in agar, but the structure is not yet elucidated.

Pectins : These are abundant in fruits, particularly in the rind of citrus fruits like oranges and lemons. They are present in the cell wall and in the intercellular substance. They contain arabinose, galactose and galacturonic acid. Pectic acid is homopolymer of the *methyl ester of* D-*galacturonic acid.*

Galactan : It has been obtained from snails.

Mannans : The cell wall of yeasts contain *mannans* which are *mannose* polymers. The main chain consists of mannose units linked by α-1, 6. Branches of one to three mannose units are joined in α-1, 5 or α-1, 3 linkage.

Xylan : In addition to cellulose all plants contain *xylan*. Xylan is a hemicellulose and consists of D-*xylose* units linked through β-1, 4 glycosidic bonds.

Inulin : It is the reserve carbohydrate of many plants. It consists of 30 *fructose* units linked β-1, 2 and has a molecular weight of about 5,000. Fructose , a 6-carbon sugar, is present in the 5-membered furanose ring form in inulin.

This polysaccharide occurs in the tubers of dehlia and bulbs of onion and garlic. The structure consists of β, 1 → 2 linkage between neighbouring fructose units. It cannot be utilised as food for man.

B. Heteropolysaccharides (heteroglycans) : There are two major groups of heteropolysaccharides. One group consists of *neutral sugars* and the other group includes *mucopolysaccharides.* The polysaccharides of the first group yield more than one type of sugar on hydrolysis, and sometimes non-sugar components also. This group includes some *hemicelluloses,* some *gums, mucilages and pectin substances.*

Neutral Sugars : Hemicellulose is found in association with cellulose in cell walls. The commonly found sugars in hemicellulose are *D-xylose, L-arabinose, D-galactose, L-rhamnose, D-mannose,* and *D-glucuronic acid.*

Gums : These are substances exuded by plants on mechanical injury or after bacterial, fungal or insect attack. The viscous substances seals the wound and thus protects the plant. Gums often contain polysaccharides, which are usually highly branched. The commercial Gum-Arabic occurs as a salt. Acidification of an aqueous solution of the gum yields the free *arabic acid.* Hydrolysis of arabic acid yields various sugars like *L-arabinose, 3-β-D-galactopyranosyl-L-arabinose; D-galactose, D-glucuronic acid* and 6-β *D-glucuronopyranosyl-D-galactose.*

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Mucopolysaccharides : These consist of *amino sugars uronic acids,* and some of their *derivatives*. They are gelatinuous substances with high molecular weight (up to 5 million). They are structural polysaccharides and are found mainly in the connective tissue of animals and as blood components. The mucopolysaccharides include *hyaluronic acid, chondroitin sulphates* and *heparin.*

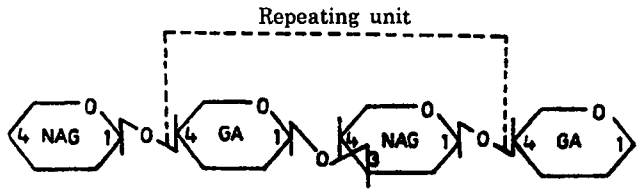
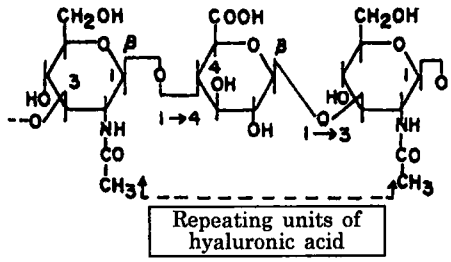


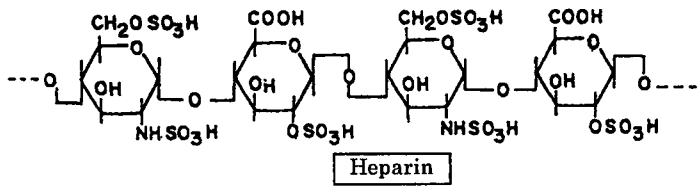
Fig. 6.20. Repeating unit of hyaluronic acid. GA, D-glucuronic acid NAG. N-acetyl glucosamine. GA is linked to NAG by β-1,3 bond to form the disaccharides which are linked by β-1,4 glycosidic bonds.

Hyaluronic acid (Fig. 6.20). It is the simplest mucopolysaccharide. it is a linear polymer of disaccharides which form the repeating unit. Each disaccharide is linked to the next by β-1,4 *glycosidic bonds*. It consists of two monosaccharides, *D-glucuronic acid* and *N-acetyl-D-glucosamine* linked by β 1,3. Hyaluronic acid is found in the skin, vitreous humour of the eye, the umbilical cord, as a coating around the ovum and in certain bacteria. It acts as a cementing substance in connective tissue. It is also present in the synovial fluid of joints, where its viscosity makes it a good lubricant.

Chondroitin sulphates are predominant in cornea, cartilage, tendons, skin, heart, valves and saliva. The repeating unit is a *disaccharide* consisting of *glucuronic acid* linked to *sulphate ester of N-acetyl galactosamine* through a β-1, 3 glycosipic bond. The disaccharides are linked through β-1,4 linkage. The sulphate esters are of two types, *chondroitin-4-sulphate (A)* and *chondroitin-6-sulphate (C).* A third type of sulphate contains *iduronic acid* instead of glucuronic acid and is labelled B.



Heparin is an anticoagulant secreted by mast cells in the intestinal mucosa, liver, lung, spleen and kidney. It is used to prevent clotting of blood. Heparin is a polymer of *glucuronic acid* and *N-acetyglucosamine.*



Other Heteropolysaccharides

Glycoproteins : These consist of *carbohydrate* linked to a *peptide* portion by *covalent* bonds only. *Mucopolysaccharides may associate* with peptides by *ionic* as well as by *covalent* bonds. Moreover, in contrast to mucopolysaccharides, glycoproteins contain no uronic acids and only a few sulphate esters. The glucoproteins include some *plasma, proteins, blood group substances* and *mucoproteins.*

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Plasma Proteins : These include *lipoproteins* and *glycoproteins*. Among the important plasma proteins are *albumin, thyroxine binding globulin, haptoglobin, fibrinogen* and the *immunoglobins.*

Blood group polysaccharides : These are present in erythrocytes, saliva, gastric mucin, cystic fluids and other body secretions. When combined with proteins, they constitute certain antigens of erythrocytes, A, B, O, Rh, etc. and differentiate the blood groups or types. When red cells containing a specific antigen of the above types, are mixed with the specific antiserum, *i.e.*, serum containing the specific antibody, agglutination of the cells takes place. Thus, by previously working out the agglutination characteristics of erythrocytes with known antisera, it is possible to decide the proper type of blood for transfusion into a patient, so that agglutination in the body (with consequent danger) may not occur after the transfusion. Chemically, these polysaccharides contain D-glucosamine and/or D-galactosamine with one or more single sugars like glucose, fructose, etc.

Bacterial polysaccharides : Specific polysaccharides are known to be present in the capsules of different types of bacteria. These are also made up of monosaccharides, uronic acids, N-acetylated aminosugars etc.

Mucoproteins : These are characteristic of bone, connective tissue and salivary glands. *Galactosamine* and *glucosamine* are the sugars most commonly found in mucoproteins. They also contain *L-fucose* and *sialic acid.*

Murein : It is a *polysaccharide* linked to short *peptide chains.* It is found in the cell walls of many bacteria. It consists of alternating units of *N-acetyglucosamine* (NAG) and *N-acetyl muramic acid* (NAM) linked by its carboxyl group to the peptide chain consisting of four amino acids (*L-alanine, D-glutamic acid, L-lysine,* and *D-alanine*).Five *glycine* residues link each peptide chain to its neighbour. The enzyme *lysozyme* hydrolyses the chain into NAG-NAM disaccharides, resulting in the rupture of the cell wall.

6.10 Compounds Similar to Carbohydrates

There are compounds which may not be sugars, but have similar configurations with important biochemical interest. Most important of them are noted below.

Inositol : It is a cyclic alcohol with 6 OH-groups (Fig. 6.21). It is considered to be a vitamin and occurs in phytic acid which is its hexa-phosphoric ester. It is also a component of some phospholipids.

Ascorbic acid : It is also known as vitamin C and may be considered as a sugar acid derived from L-glucose; L-ascorbic acid on mild oxidation becomes dehydroascorbic acid. Both forms are biologically active. (Fig. 6.21).

Sialic acids : These are acetyl derivatives of a 9-carbon 3-deoxy-5-amino sugar called neuraminic acid (Fig. 6.21).

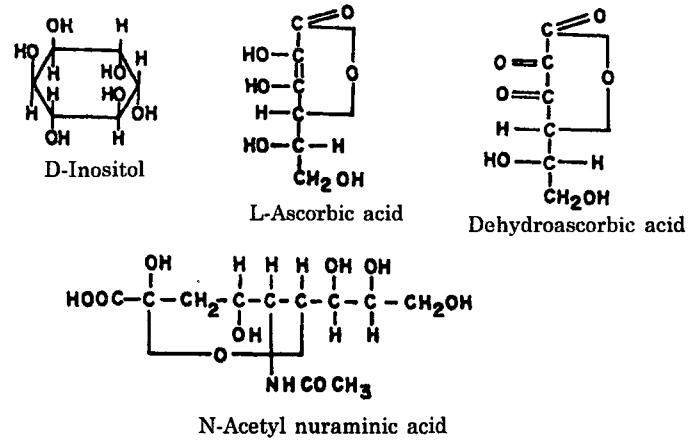

Fig. 6.21. Structures of some compounds similar to carbohydrates.

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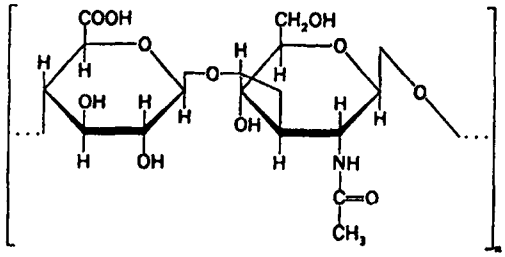
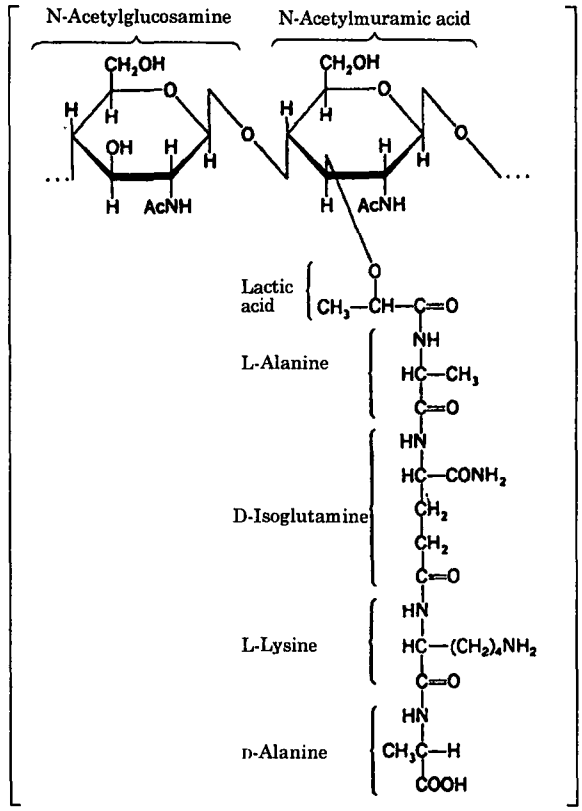
6.11 Polysaccharides in Cell Walls

Animal cells do not possess a well-defined cell wall but have a *cell coat*, visible in the electron microscope, that plays an important role in the interaction with adjacent cells. These cell coats contain glycoproteins, glycolipids, and mucopolysaccharides. The mucopolysaccharides are gelatinous substances of high molecular weights (up to 5×10^6) that both lubricate and serve as a sticky cement. One common mucopolysaccharide is hyaluronic acid, a heteropolysaccharide, composed of alternating units of D-glucuronic acid and *N*-acetyl-D-glucosamine. The two different monosaccharides are linked by a $\beta(1 \rightarrow 3)$ bond to form a disaccharide that is linked $\beta(1 \rightarrow 4)$ to the next repeating unit. Hyaluronic acid, found in the vitreous humor of the eye and the umbilical cord, is water soluble but forms viscous solutions.



Hyaluronic acid unit

Chondroitin, similar in structure to hyaluronic acid except that the amino sugar is *N*-acetyl-D-galactosamine, is also a component of cell coats. Sulfate esters (at the C-4 or C-6 positions of the amino sugar) of chondrotin are major structural components of cartilage, tendons, and bones.



Repeating unit of peptidoglycan of Staphylococcus *aureus*

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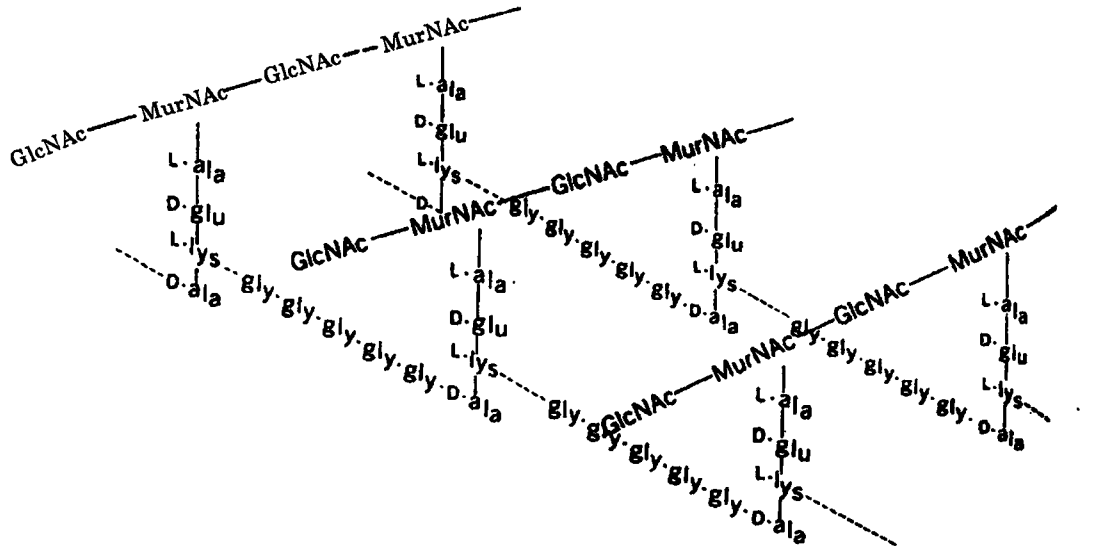


Fig. 6.22. The linear chains of the peptidoglycan are cross-linked by glycine pentapeptides.

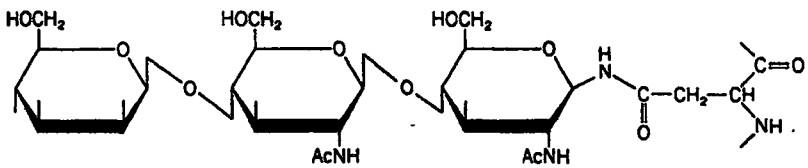
Bacterial cell walls, which determine many of the physiological characteristics of the organism they enclose, contain a heteropolysaccharide linked to a short chain of amino acids. Since the individual chains of amino acids are not as long as in proteins, such polymers have been termed peptidoglycans rather than glycoproteins. The heteropolysaccharide is an alternating chain of *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) joined by a $\beta(1 \rightarrow 4)$ glycosidic bond. *N*-acetylmuramic acid consists of a *N*-acetyl glucosamine unit that has its C-3 hydroxyl group joined to the α-hydroxyl group of lactic acid by an ether linkage. In the peptidoglycan, the carboxyl group of each lactic acid unit is linked, in turn, to a tetrapeptide that usually contains both D-alanine and L-alanine. Other amino acids found in the tetrapeptide may include D-glutamine, D-*iso*-glutamine, L-lysine, or diaminopimelic acid.

The linear polysaccharide chain of the peptidoglycan has a tetrapeptide branch at every second hexoseamine unit that is cross-linked to adjacent, parallel, polysaccharide chains. In the cross-linking (Figure 6.22), the carboxyl group of the terminal D-alanine moiety is attached to a pentaglycine residue that, in turn, is attached to the ε-amino group of lysine in the next adjacent glycan unit.

The antibiotic activity of penicillin is due to its ability to inhibit the last step in the biosynthesis of bacterial peptidoglycans. With the synthesis of this essential component of the cell wall inhibited, the bacteria are unable to grow or replicate.

6.12 Glycoproteins

Most of the oligo-and polysaccharides in the animal and plant cell are linked covalently to protein or lipid molecules known as glycoproteins or glycolipids. In many glycoproteins, the amide group of asparagine is linked through an *N*-glycosyl bond to a core trisaccharide consisting of one molecule of mannose (Man) and two molecules of *N*-acetyl glucosamine (GlcNAc).



Manβ1, 5GlcNAcβ1, 4GlcNacβ-Asn

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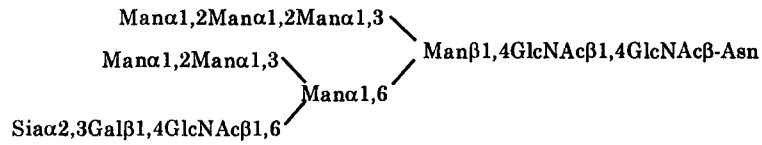
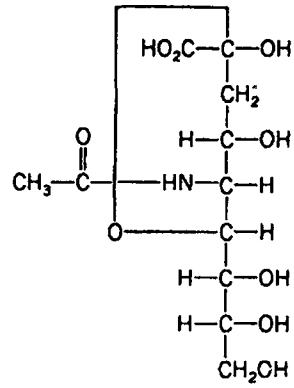
The mannose moiety of the core polysaccharide constitutes a branch point where two more mannose molecules are linked in α(1 → 3) and α(1 → 6) linkages. Either or both of the two mannose units may then serve as additional branch points for further enlargement of the polysaccharide component. In a highly branched example, galactose, sialic acid, and *N*-acetyl glucose are additional components of the branched polysaccharide.



A sialic acid is a ketose containing nine carbon atoms (ketononose) that may be acylated with acetic or glycolic acid. *N*-acetyl-D-neuraminic acid is a specific example of a sialic acid.



N-Acetyl-D-neuraminic acid

The core polysaccharide can also be linked to the protein component through an *O*-glycosyl bond to the hydroxyl group of serine instead of the asparagine amide group. Hydroxylysine can substitute for serine, and other sugars such as xylose and galactose can substitute for the GlcNAc. The latter are found in such glycoproteins as collagen and proteoglycan. It is apparent that glycoproteins, which are widely distributed in all living matter, can show considerable diversity.

Much remains to be learned of the structure of cell walls before we can completely understand such important phenomena as the immune response and cellular growth and differentiation.

6.13 Sweetness of Sugars

The word sugar is synonymous to sweetness. Different sugars vary in their extent of sweetness. Surprisingly, even for sweetness there is a standard scale where *sucrose* (*cane sugar; table sugar*) has been *assigned a value 100.*

On the other hand, in comparison to sucrose :

(*i*) Glucose is *less sweet* (value on sweetness scale—75)

(*ii*) Fructose is *more sweet* (value on sweetness scale—173)

Honey, Candies, Jellies and Fructose

Fructose is the sweetness of all simple sugars. One of the characteristics of fructose is that it *cannot be crystallized from solution.* Although glucose and sucrose can be readily crystallized from solutions but presence of fructose prevents their crystallization. It should noted that

(*i*) Honey contains more than 50% fructose, which is also responsible for its extremely sweet taste.

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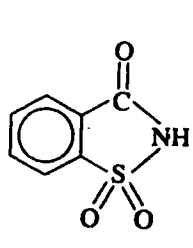
(*ii*) Honey does not crystallize even if kept over long periods of time because of high contents of fructose that prevent crystallization.

(*iii*) Invert sugar (glucose and fructose in I : I ratio) is added to candies and jellies to prevent crystallization and development of undesirable grainy texture.

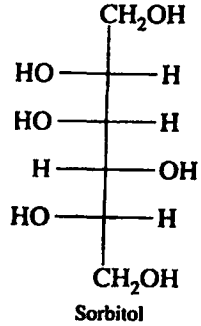
Sweetness without Sugars (Artificial Sweeteners)

Some compounds which are not sugars happen to be much sweeter than glucose, fructose, or sucrose. Unlike sugars these compounds do not have any food value and have very low calories. Mainly diabetic patients use such compounds as substitutes for sugar. A few examples of non-sugar sweeteners are *saccharin, sorbitol,* and *aspartame.* Note that

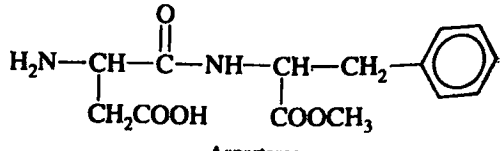
- Saccharin is 300 *times sweeter than sucrose.*
- Sorbitol is a polyhydroxy compound and its sweetness is *nearly* 60% *of that of sucrose.* It is used as a sweetening agent by diabetic patients as its metabolism is insulin independent. One of the advantages of sorbitol is that it is stable at higher temperatures (upto 150°C) and can be used effectively during cooking and baking. It is to be noted that sorbitol gives nearly the same calories as sucrose.
- Aspartame is a methyl ester of *aspartylphenylalanine* and is 200 *times sweeter than sucrose.*



Saccharin



Sorbitol



Aspartame

Nowadays, aspartame (a dipeptide derivative) is marketed widely as 'sugar free tablets' to lure the calorie conscious people. The low calorie drinks available in the market also contain aspartame as a sweetener.

6.14 Functions of Carbohydrates

The most important function of carbohydrates is to provide *energy* to the body. They are also *structural components* of tissues. Other functions include *regulation of fat metalism, protein sparing functions,* and function in the *digestive tract.*

1. Storage substances of potential energy : Carbohydrates are storage substances of potential energy in animals. About 60% of the total energy requirement of man is provided by the breakdown of carbohydrates. One gram of carbohydrate on oxidation yields on an average four calories. Glucose supplies the immediate energy needed by tissues. Glucose is the sole form of energy for the brain and other nervous tissue. Lack of glucose, or of oxygen for its metabolism, leads to rapid damage to the brain. Carbohydrate is stored in the body in the form of *glycogen.* About 100 gm of glycogen is stored in the liver and by its breakdown maintains the glucose level in the blood. Muscles contain about 200 to 240 gm glycogen. This glycogen is, however, utilized only by the muscles and is not available for regulating the blood sugar level.

2. Structural component : Carbohydrates are important components of some structural materials of living organisms. *Monosaccharides* are important constituents of nucleic acids,

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coenzymes, flavoproetins and blood group substances. *Vitamin C* (ascorbic acid) is related to sugars. *Immunopolysaccharides* play a part in the resistance of infections. *Hyaluronic acid* is the viscous substance in the matrix of connective tissue. *Heparin* prevents the clotting of blood. *Glucuronic acid,* which occurs in the liver, acts as a detoxifying agent by combining with toxic substances and bacterial by-products. *Chondroitin* sulphates are found in cornea, cartilage tenders, skin, heart valves and saliva. *Glycosides* are components of steroid hormones. *galactolipins* are constituents of nervous tissue. The cell wall of plants and the polysaccharides in the capsule of bacteria are carbohydrates.

3. Regulation of fat metabolism : Some carbohydrates are essential for normal oxidation of fats. When carbohydrates are restricted in the diet there is more rapid metabolization of fats. This results in the accumulation of incompletely oxidized intermediate products leading to *ketosis*. This is common in uncontrolled diabetes mellitus.

4. Protein sparing function : Carbohydrate is preferentially metabolized in the body as a source of energy as long as it is present in the required quantity. This spares protein for building of tissue. When there is deficiency of calories in the diet, however, fat and then protein are utilized for supplying energy.

5. Role in gastrointestinal function : Indigestible substances like cellulose, hemicellulose and pectins provide the bulk or roughase of food, and thus help the peristaltic movements of the digestive tract. Lactose promotes the growth of desirable bacteria in the small intestine. These bacteria synthesize certain-B-complex vitamins. Lactose also increases calcium absorption.

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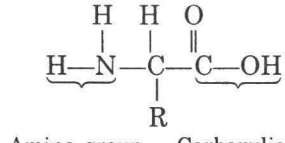
7 Chemistry of Amino Acids

7.1 Introduction to Amino Acids

Amino acids are the building blocks of which proteins are made up of. Simple proteins contain only amino acids while conjugated proteins have additional components.

In principle, the term *"amino acids"* could be used to refer to any compound containing an amino group and acidic function. In actual practice, this term is often used with reference to α-amino carboxylic acids which are isolated from natural sources. α-Amino acids have the following general structure :



By convention, the carbon atom to which the carboxylic group is attached is called an alpha (α) carbon.

7.2 Essential, Semi-essential and Non-essential Amino Acids

1. Essential amino acids : These are the amino acids which are not synthesised in the body and have to be provided in the diet. They are also called indispensable amino acids. The deficiency

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of essential amino acids may cause various diseases like nervous breakdown, inhibition of full mental growth and their continuous deficiency may even cause death in young animals.

There are eight essential amino acids which owe special importance to human beings. These are valine, leucine, isoleucine, threonine, methionine, phenylalanine, tryptophan and lysine.

Adequate amounts of essential amino acids are required to maintain the proper nitrogen balance.

2. Semi-essential amino acids : These are the amino acids which are synthesised partially by the body but not at the rate to meet the requirement of the body. Examples of semiessential amino acids are arginine and histidine.

3. Non-essential amino acids : These are the amino acids which are synthesised by the body. These amino acids are derived from carbon skeletons of lipids and carbohydrates metabolism or from the transformation of essential amino acids.

Non-essential acids may be synthesized in individual cells from simple starting materials containing carbon, hydrogen, oxygen and nitrogen. In human beings, there are twelve non-essential amino acids which have special significance. These are alanine, arginine, aspartic acid, aparagine, cystine, cysteine, glutamic acid, glutamine, glycine, histidine, proline and serine.

7.3 Number of Amino Acids

More than 200 amino acids have been isolated and identified but only 25 are obtained upon hydrolysis of typical proteins. Out of 25, only 20 amino acids are of general occurrence because they are usually found in all proteins. Again, 10 out of 25 amino acids are essential amino acids. Also, 23 out of 25 amino acids are α-amino acids whereas remaining 2, *i.e.,* proline and hydroxyproline, are imino acids.

Twenty amino acids are *specified by the genetic code* and are utilized in ribosomes. They are :

Glycine	Serine	Asparagine	Histidine
Alanine	Threonine	Glutamic acid	Phenylalanine
Valine	Cysteine	Glutamine	Tyrosine
Leucine	Methionine	Arginine	Tryptophan
Isoleucine	Aspartic acid	Lysine	Proline

The structures of these twenty acids are given in Table 7.1.

Some additional of these twenty acids are known which have been isolated from some proteins. These are *modifications of the amino acids specified by the genetic code.* Some of these are as follows :

(*i*) *Cystine :* It is a double amino acid which is formed by linking of two *cysteine* molecules by a disulphide kinkage (—S—S—).

(*ii*) *Hydroxyproline :* It is formed by the addition of hydroxyl (—OH) group to *proline* after the protein has been synthesized.

(*iii*) *Hydroxylysine :* It is similarly formed by the addition of a hydroxyl group to *lysine.* Hydroxyproline and hydroxylysine occur only in *collagen* and *gelatin.*

(*iv*) *Thyroxine :* It is formed from *tyrosine* in the thyroid gland and found only in the protein *thyroglobulin* elaborated from the gland.

(*v*) *Diaminopimelic acid :* It is a constituent of the mucopeptide of bacterial cell walls.

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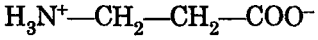
(*vi*) *Desmosine and isodesmosine :* These are the modifications of lysine found in *elastin.*

(*vii*) *Mono-, di-, and tri-methyl lysines :* These are the modification of *lysine* which are found in *histones and myosine.*

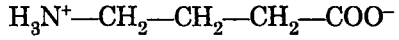
(*viii*) *3-Methyl histidine :* It is a modification of *histidine* which is found in certain types of *Myosine.*

In addition to the amino acids which are constituents of proteins more than 200 *non-protein amino acids* have been isolated from natural sources. Many of these are of physiological importance and are found as metabolites. They include :

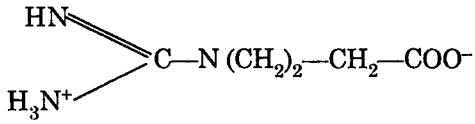
(*i*) β-*Alanine :* It is a component of *coenzyme* A and the vitamin *pantothenic acid* and is produced in *pyrimidine catabolism.*



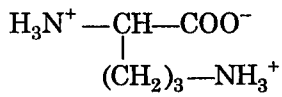
(*ii*) γ-*Amino-butyric acid (GABA) :* It is formed in the brain by decarboxylation of glutamic acid. It is an inhibitory trasmitter in the central nervous system.



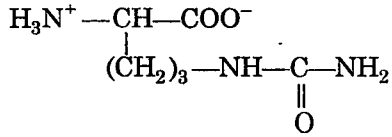
(*iii*) *Creatine :* It is an important constituent of muscles.



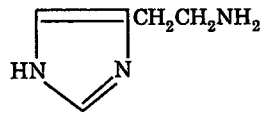
(*iv*) *Ornithine :* It is an intermediate in the biosynthesis of urea.



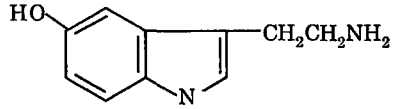
(*v*) *Citrulline :* It is another intermediate in the biosynthesis of urea. It is the precursor of *arginine* which is the end product of protein catabolism.



(*vi*) *Histamine :* It is a vasodilator which is involved in shock and allergic reactions. It is a decarboxylation product of *histidine.*



(*vii*) *Serotinin (5-hydroxytryptamine) :* It is important in the transmission of nerve impulses. It is a powerful vasoconstrictor and stimulates the contraction of smooth muscle. It occurs in the brain, the gut and blood plates and is synthesized from *tryptophan.*



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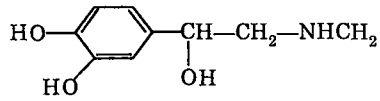
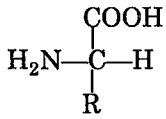
(*viii*) *Epinephrine (adrenalin) :* It is a derivative of *tyrosine*. It plays an important role in many regulatory processes.



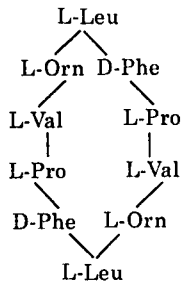
7.4 L-α-Amino Acids

All the natural amino acids that occur as constituents of proteins (with the exception of glycine) have a centre of asymmetry at the α-position, *i.e.,* they are optically active. Although some of the amino acids found in proteins are dextrorotatory and some are laevorotatory at pH 7.0 all have absolute configuration comparable to that of L-glyceraldehyde and hence are L-α-amino acids corresponding to the projection formula shown as follows :



L-Amino acids

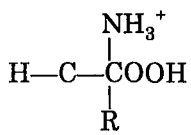
Although L-amino acids, exclusively, are found in proteins, naturally occurring D-amino acids have been found in a few sources. For example, D-phenylalanine residues by Russian scientists (hence S for Soviet), and D-valine occur in actinomycin-D, a potent inhibitor of RNA synthesis. D-alanine and D-glutamic acid are found in the peptidoglycan of the cell wall of gram-positive bacteria.



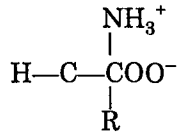
Gramicidin-S

7.5 Ionisation of Amino Acids

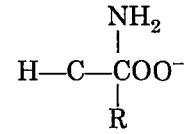
At *intermediate pH values of* about 6.0, the *α-carboxyl group* of the amino acid becomes *negatively* charged and the *α-amino* group *positively* charged. As the amino acid molecule simultaneously has a negative (COO^-) and a positive (NH_3^+) group, there is little or no net charge. Such ions are termed as *zwitterions* or *dipolar ions*. The point at which the molecule is having equal positive and negative charges is termed as the *isoelectric point*. At this point the amino acid does not migrate in an electric field. If the pH is decreased, the—COOH group ceases to be ionized, and the molecule gets *positive* charge :



Predominant form at pH 1



Prodominant form at pH 6



Predominant form at pH 11

Increasing the pH causes the NH_3^+ group to dissociate and loses a proton, and it becomes $—NH_2$. The molecule as a whole thus gets *negative* charge because of the negative charge on the $—COO^-$ group.

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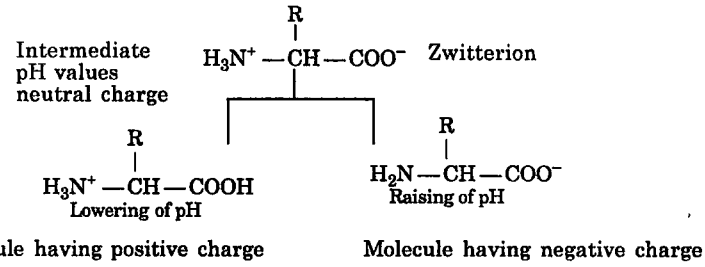
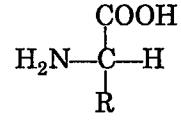


7.6 Stereochemistry (Optical Isomerism) of α-Amino Acids

All the naturally occurring α-amino acids, with the exception of glycine, possess at least one asymmetric carbon atom and exhibit inversional isomerism. All of the naturally occurring α-amino acids have the following configuration about the α-carbon atom.



Alanine (where R = CH_3) has one asymmetric carbon atom and can exist in two isomeric forms. These two isomers are similar in all respect except the sign of rotation of the plane polarised light, one rotating to the right and the other to the left. These forms are related to each other as an object to its mirror image as depicted for alanine in Fig. 7.1.

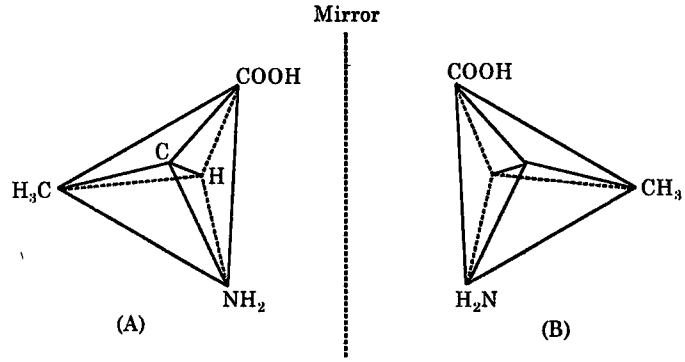


Fig. 7.1. Three-dimensional representation of the enantiomeric forms of alanine.

The dextrorotatory alanine is arbitrarily identified with model A and laevorotatory with model B.

The amino acids *isoleucine threonine, hydroxylysine and 4-hydroxylysine* have two asymmetric carbon atoms each, and hence occur in four isomeric forms.

Amino acids can occur in two isomeric forms, *dextrorotatory* (D form) and *laevorotatory* (L form), which are mirror images of each other. The direction of rotation of polarized light need not be necessarily to the right in D forms and to the left in L forms. The designations L and D are used with reference to be spatial relationship with serine which in turn is related to *glyceraldehyde,* the parent compound of the carbohydrate group.

The natural (–) serine has been selected as reference compound for correlating the configurations of amino acids, the relationship to this acid is indicated by Ds or Ls. It has been proved that Lg ≡ Ls, *i.e.,* natural serine belongs to the L-series. This is similar to D-glyceraldehyde which is chosen as absolute standard for carbohydrates. All amino acids which can be converted

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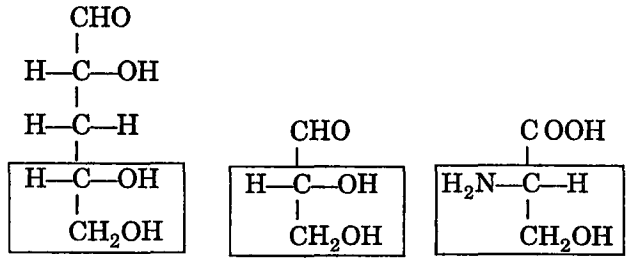
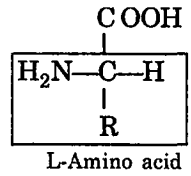
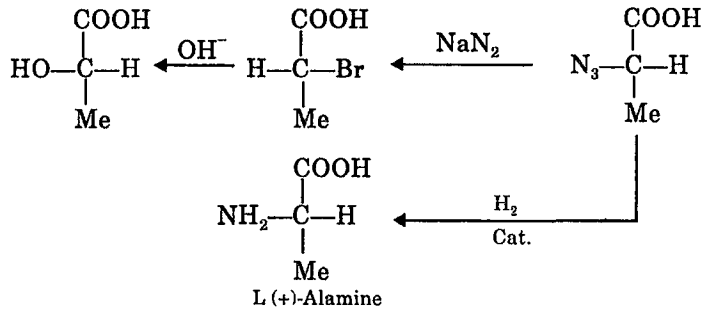


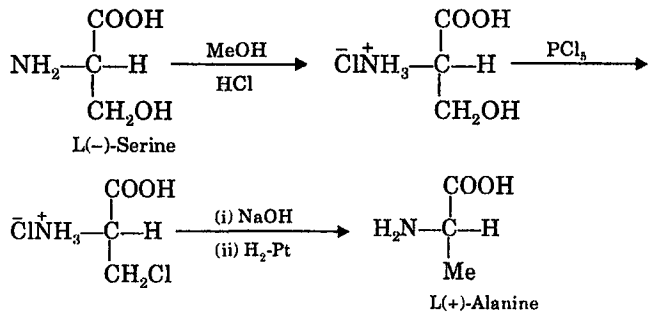


or obtained from L-serine are assigned as laevorotatory. The correlation between the two standards was established by the following method :

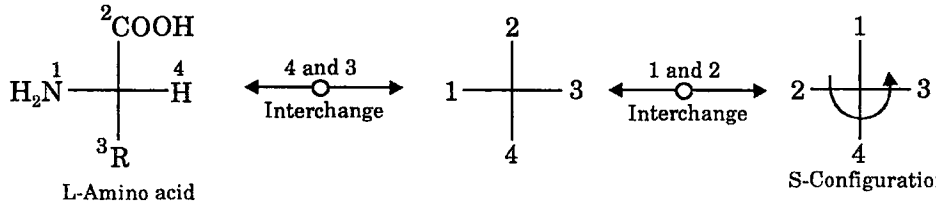
(*i*)



(*ii*)



The *rectus and sinister* (R and S) system may be used to designate the absolute configuration about the asymmetric carbon atom. According to this system, the L-amino acids exhibit S configuration, because priority order for assigning configuration is $NH_2 > COOH > R > H$.



Priority order for assigning configuration is $NH_2 > COOH > R > H$.

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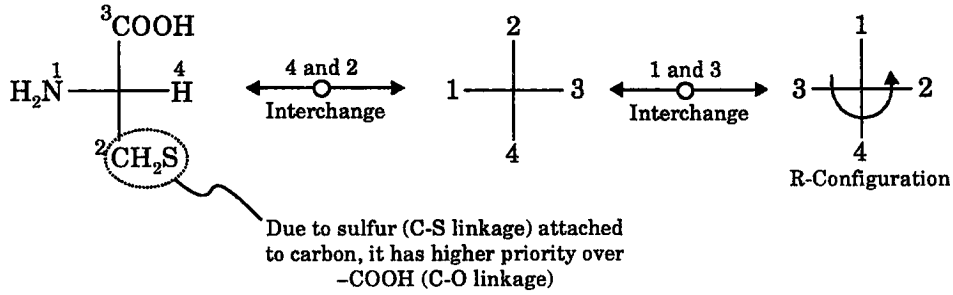
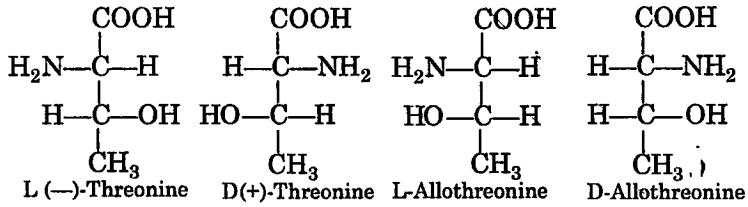
Exceptions to this are cystine and cysteine, where sulfur is present in the side chain (R) and thus in assigning the configuration, the side chain (containing sulfur) gets higher priority over –COOH group, (R > COOH).



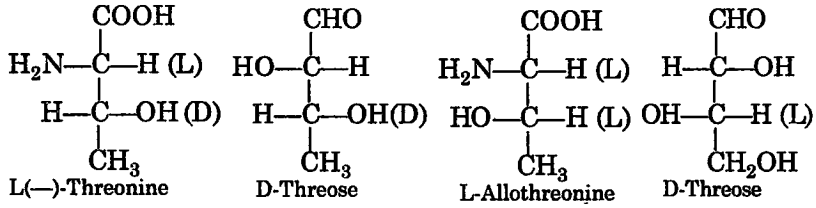
Priority order for assigning configuration is NH_2 > CH_2S- > COOH > H

Some of the naturally occurring amino acids contain more than one asymmetric centre and can exist in more than two stereoisomeric forms. For example, threonine, isoleucine and hydroxyproline exist in four stereoisomeric forms. Apart from the L and D forms, two allo-modifications (L and D) are known which have opposite configuration only at one asymmetric carbon atom relative to the former two molecules. Compounds which bear such a relation to each other are referred to *as diastereomers*. They differ in physical properties and also exhibit differences in their quantitative chemical behaviour (reaction rates). The four isomers of threonine are as follows :



If we examine the above formula carefully, we observe that L-acids belong to the L-series of amino acids because the α-carbon is configurationally related to L (+)-alanine. However, the second chiral centre in these formula is configurationally related to the sugar series.

In the structures given below, the L-series of amino acids has been correlated with L-glyceraldehyde and hence the configuration of the α-carbon atom is the absolute one.



In the rectus and sinister system, L-threonine would be designated as (2S : 3R) threonine or (2S, 3R)-2-amino-3-hydroxybutanoic acid and L-allothreonine as (2S, 3S)-2-amino-3 hydroxybutanoic acid.

Almost all naturally occurring amino acids have the L form. D forms are found to occur naturally in some cases (over 20 are known), *e.g.*, in antibiotics made by some micro organisms

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and in bacterial cell walls they are not, however found in proteins, as only the L forms are used in protein synthesis.

7.7 Nomenclature of Amino Acids

Amino acids may be named analogously to the hydroxy acids with the amino group designated as α, β, λ, δ etc., to indicate its position on the chain of the carboxylic acid, which is called by its common name.

In the IUPAC system, these compounds are named as amino derivatives of the corresponding acid, with the position of the amino group defined by an appropriate number. Note however, that the α — C is C — 2, the β — C is C — 3, etc.

7.8 Classification of Amino Acids

There are three main schemes for classifying amino acids :

Scheme I : On the basis of charge, amino acids may be classified as neutral, basic or acidic.

(a) Neutral amino acids : These contain one amino-group and one carboxylic group.

(b) Acidic amino acids : These contain one amino group and two carboxylic groups.

(c) Basic amino acids : These contain two amino groups and one carboxylic group.

This classification of amino acids along with their respective structures are given in Table 7.1.

Table 7.1 enlists the natural amino acids along with their classification, structure, and abbreviations (three letter and one letter code).

Table 28.1
NATURAL AMINO ACIDS

Name	*Abbreviation*		†*Structure at pH = 7.0*	*Isoelectic point [pI]*
	Three letter code	*One letter code*		
1	*2*	*3*	*4*	*5*
Neutral amino acids (with nonpolar side chains)				
@Glycine	Gly	G	$H-\underset{\displaystyle NH_3^+}{\underset{\vert}{C}}HCO_2^-$	6.0
Alanine	Ala	A	$CH_3-\underset{\displaystyle NH_3^+}{\underset{\vert}{C}}HCO_2^-$	6.0
Valine*	Val	V	$(CH_3)_2CH-\underset{\displaystyle NH_3^+}{\underset{\vert}{C}}HCO_2^-$	6.0
Leucine*	Leu	L	$(CH_3)_2CHCH_2-\underset{\displaystyle NH_3^+}{\underset{\vert}{C}}HCO_2^-$	6.0
Isoleucine*	Ile	I	$CH_3CH_2\underset{\displaystyle CH_3}{\underset{\vert}{C}}H-\underset{\displaystyle NH_3^+}{\underset{\vert}{C}}HCO_2^-$	6.0

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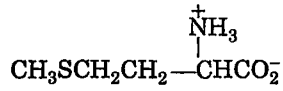
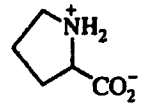
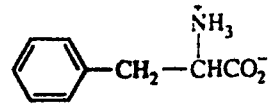
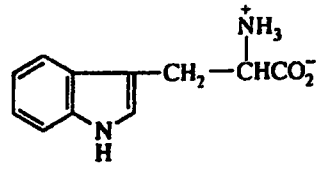
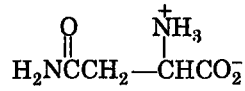
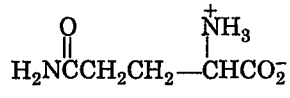
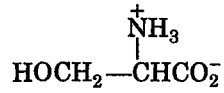
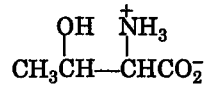
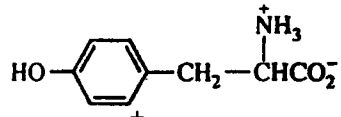
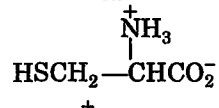
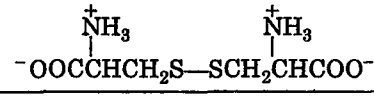
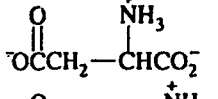
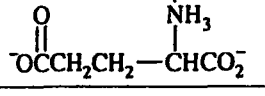
1	*2*	*3*	*4*	*5*
Methionine*	Met	M		5.7
@@Proline	Pro	P		6.3
Phenylalanine*	Phe	F		5.5
Tryptophan*	Trp	W		5.9
Neutral amino acids (with polar, but nonionized side chains)				
Asparagine	Asn	N		5.4
Glutamine	Gln	Q		5.7
Serine	Ser	S		5.7
Threonine*	Thr	T		5.6
Tyrosine	Tyr	Y		5.7
Cysteine	Cys	C		5.1
‡Cystine	Cys-Cys			
Acidic amino acids (side chain with carboxylic acid group)				
Aspartic acid	Asp	D		2.8
Glutamic acid	Glu	E		3.2

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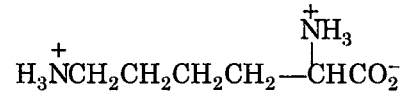
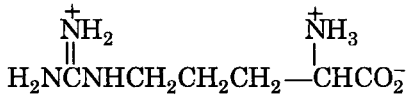
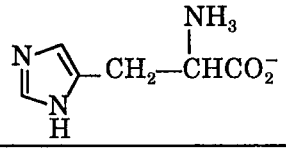
1	2	3	4	5
Basic amino acids (side chain with nitrogenous basic group)				
Lysine*	Lys	K		9.7
Arginine*	Arg	R		10.8
Histidine*	His	H		7.6

Notes :

* Amino acids with an asterisk are essential amino acids, that must be supplemented through diet.

† At pH = 7, Asp and Glu have a net negative charge and exist as anions. At pH = 7, Lys and Arg have a net positive charge and exist as cations. Rest of the amino acids at this pH exist in the neutral form.

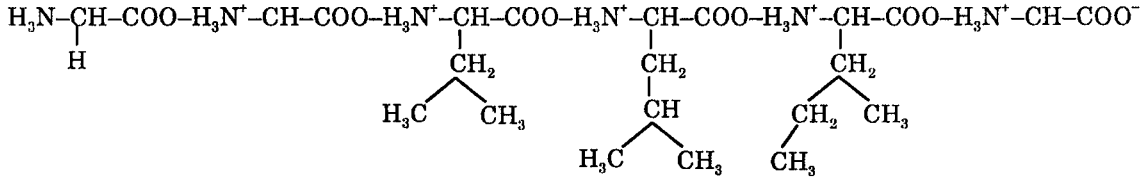
‡ Structurally, in cystine, the two cysteine molecules are joined through sulfur (disulfide linkage).

@@ Proline is an α-imino acid.

@ Except Glycine all other amino acids are optically active.

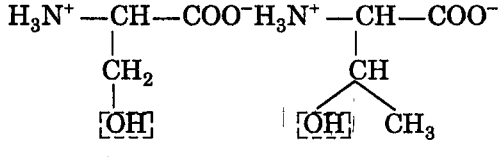
Scheme II : Another scheme for the classification of amino acids is based on the structure of side chains. On the basis of the structure of side chain, amino acids are divided into seven groups.

Group I : It *includes aliphatic amino acids with aliphatic* side chains : glycine, alanine, valine, leucine and isoleucine. The structures of these amino acids are as follows :



Glycine (gly) Alanine (ala) Valine (val) Leucine (leu) Isoleucine (ile)

Group II : It includes *hydroxyamino acids* with side chains containing *hydroxyl (–OH) groups* : serine and threonine. The structures of serine and threonine are given as follows :



Serine (ser) Threonine (thr)

Group III : It includes *sulphur containing amino acids,* with side chains containing sulphur atoms : cysteine and methionine. The structures of these sides chains are as follows :

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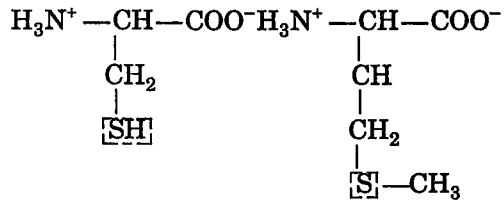
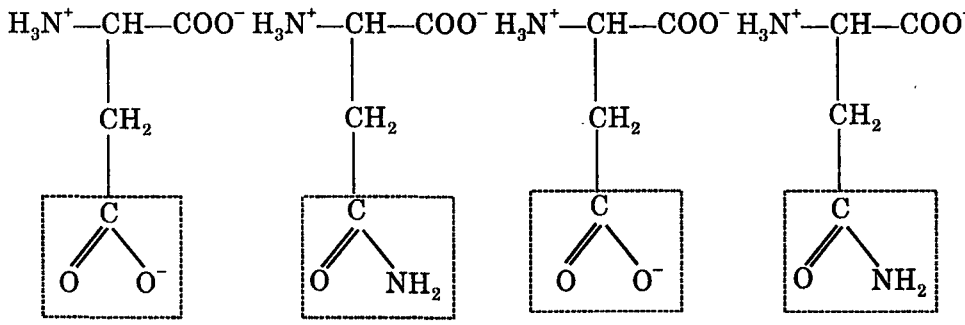


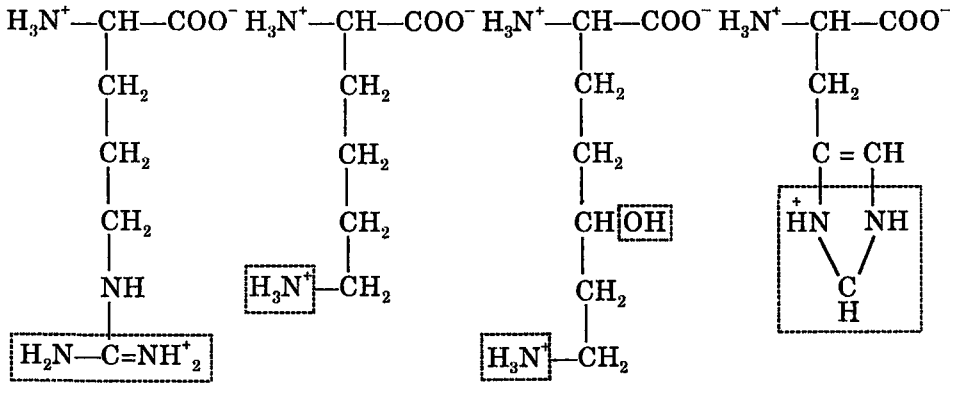
Cysteine (Cys) Methionine (met)

Group IV : It includes *dicarboxylic amino acids and their amides,* with side groups containing acidic (carboxylic) groups of their amides : aspartic acid (aspartate) asparagine, glutamic acid (glutamate), and glutamine. The structures of these amino acids are as follows :



Aspartate (asp) Asparagine (asn) Glutamate (glu) Glutamine (gln)

Group V : It includes amino acids with side chains containing *basic groups* : arginine, lysine, hydroxylysine and histidine. The structures of these amino acids are given as follows : acids.



Arginine (arg) Lysine (lys) Hydroxylysine (hyl) Histidine (his)

Group VI : It includes aromatic amino acids, with side chains having aromatic rings : phenylalanine, tyrosine, tryptophan and thyroxine. (*Note :* histidine, an aromatic amino acid, has been included in Group V). The structures of these amino acids are given as follows :

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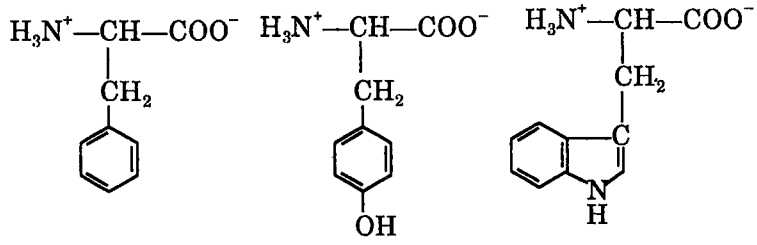
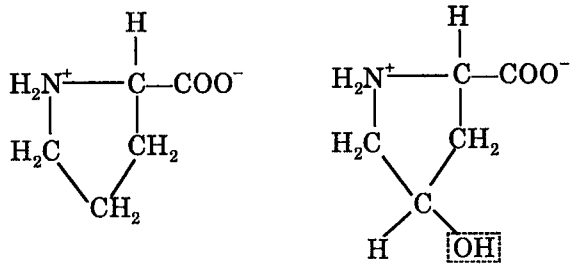


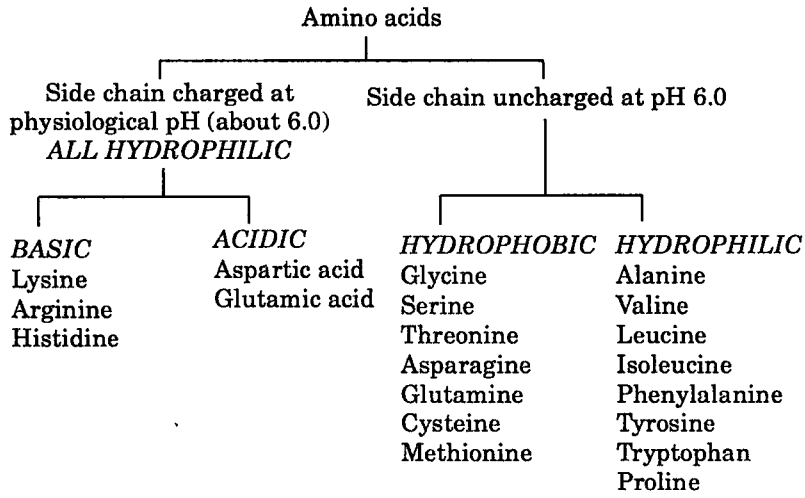
Phenylalanine (phe) Tyrosine (tyr) Tryptophan (trp)

Group VII : It includes amino acids, which are strictly speaking not amino acids as they have an imino group (NH_2^+) instead of a NH_3^+ : proline and 4-hydroxyproline. The structures of these acids are as follows :



Proline (pro) 4-Hydroxyproline (hyp)

Scheme III : On the basis of the affinity of their side chains for water, amino acids are classified as *hydrophilic* or *hydrophobic*. In *hydrophilic* amino acids the side chains have high affinity for water. Some are *charged* while others are *uncharged. Charged* side chains attract water dipoles. The side chains may be *positively charged* and *basic* (*e.g., lysine, arginine, histidine*) *or negatively charged* and *acidic.*



(*e.g., asartic acid, glutamic acid*). *Uncharged* hydrophilic side chains can readily participate in hydrogen bond formation with water. Examples of uncharged hydrophilic amino acids are *glycine, serine, threonine, asparagine, glutamine, cysteine* and *methionine.*

The side chains of *hydrophobic* amino acids do not readily interact with water. In aqueous solution, the side chains tend to have mutual attraction. The hydrophobic amino acids are *aliphatic*

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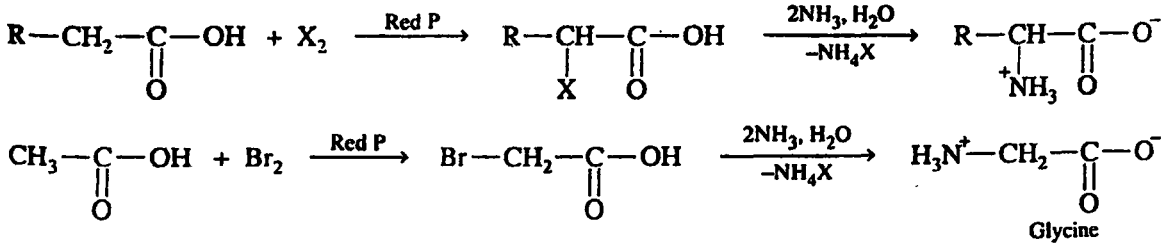
(alanine, valine, leucine and isoleucine) , and *aromatic (phenylalanine, tyrosine, tryptophan)* amino acids, in addition in the imino acids *prolone.*

Synthesis of Amino Acids

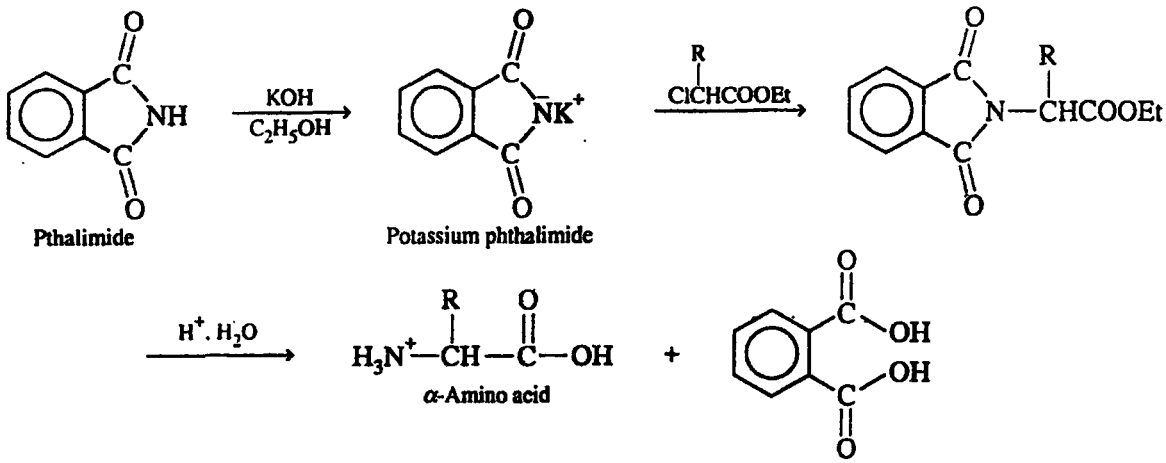
The amino acids can be synthesized by a number of methods. However, a single method cannot be used for preparation of all amino acids. The different methods used extensively for preparation of different kinds of amino acids are as follows :

(1) Direct ammonolysis of α-haloacids : The carboxylic acids can be converted to α-chloro or α-bromo acids by Hell-Volhard-Zelinsky method (HVZ reaction) using chlorine or bromine in the presence of red phosphorus. The α-bromo or α-chloro acids on treatment with excess of ammonia result in the substitution of chlorine or bromine by $-NH_2$ to give α-amino acid.

Glycine, alanine, valine, leucine, and aspartic acid can be prepared by this method.



(2) Gabriel phthalimide synthesis : Phthalimide is converted to its reactive salt by reaction with ethanolic KOH. The potassium salt of phthalimide on reaction with α-halo acids (α-haloester is preferred), followed by hydrolysis results in the formation of α-amino acids.



This method results in higher yield of amino acids as compared to that obtained by the amination of α-halo acids. Glycine and leucine are best prepared by this method.

(3) Strecker synthesis : An aldehyde on reaction with KCN in the presence of ammonia or ammonium chloride results in the formation of α-aminonitrile. Hydrolysis of α-aminonitrile in acidic medium yields an α-amino carboxylic acid. The overall reaction is known as Strecker synthesis, which basically involves conversion of an aldehyde to α-amino acid having one more carbon atom than the parent aldehyde.

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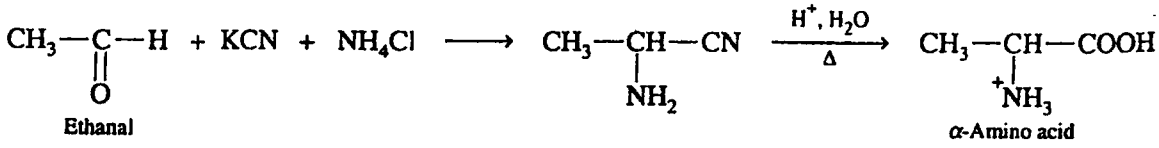
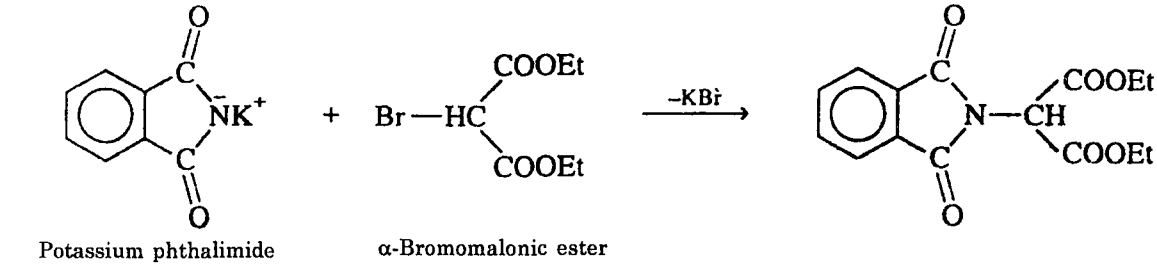
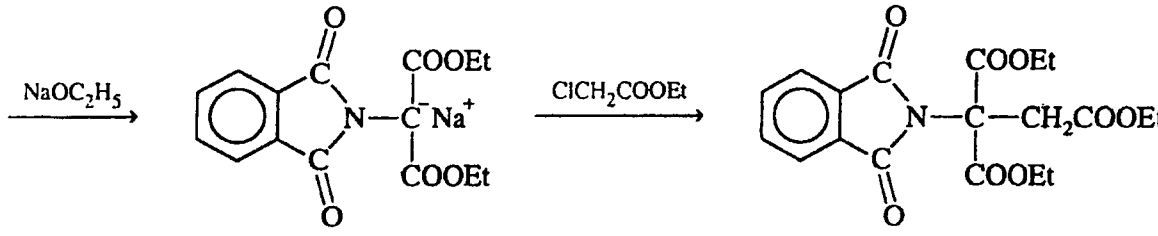
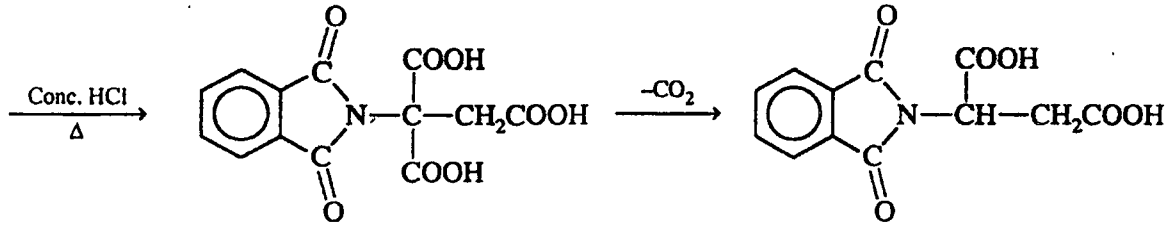
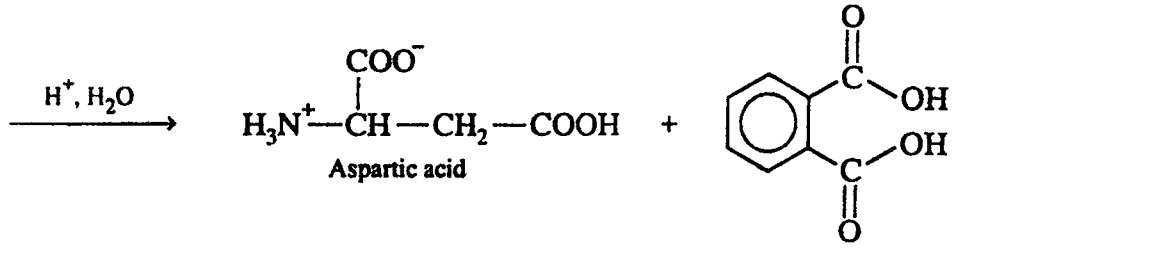


(4) Phthalimido malonic ester synthesis : This method is a modification of Gabriel phthalimide method and involves reaction of potassium phthalimide with α-bromomalonic ester to produce phthalimidomalonic ester. The sodium salt of ester on reaction with an α-haloester followed by hydrolysis results in the formation of α-amino acid. This reaction is used for the synthesis of acidic amino acids and hydroxy amino acids such as serine, glutamic acid and aspartic acid.









The methods described so far yield racemic mixture of amino acids. To obtain optically active enatiomers of amino acid, resolution of mixture is carried out in the presence of a chiral reagent.

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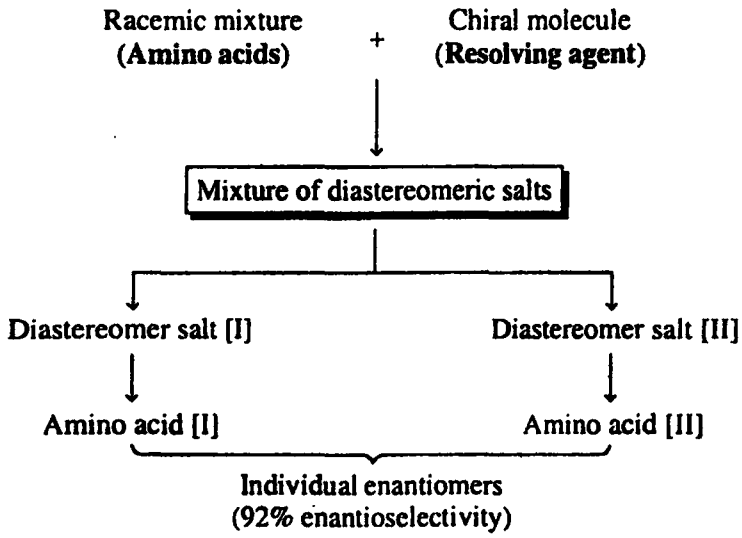
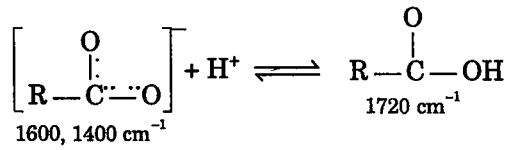


In nature, the synthesis of amino acid occurs with 100% *enantioselectivity.*

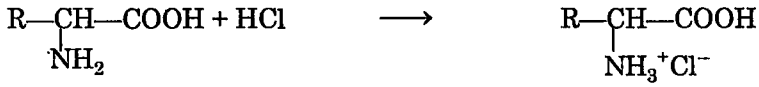
7.9 General Properties of Amino Acids

1. Physical properties : The natural α-amino acids are colourless, stable, high-melting solids. Most are soluble in water at least to some extent and are soluble in common organic solvents.

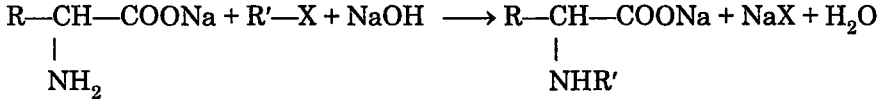
2. Infra-red spectra : Amino acids, in the solid state or in neutral solution, do not exhibit any infra-red band at 1720 cm^{-1}, a characteristic frequency of non-ionised carboxylic group. Instead, they exhibit absorption bands near 1400 and 1600 cm^{-1}, frequencies characteristic of the carboxylate ion. These also exhibit bands in the range 3130–3000, 1600–1500 and 1550–1480 cm^{-1}, characteristic of NH_3^+. These bands are inconsistent with the dipolarion structure. In strongly acidic solution of amino acids, however, the 1720 cm^{-1} absorption does appear, indicating operation of the carboxyl-carboxylate equilibrium.



3. Reactions due to amino group : (a) With strong inorganic acids : Due to the presence of a lone pair of electrons on the nitrogen, amino acids form salts with strong inorganic acids. These salts are generally sparingly soluble in water. However, free amino acids may be liberated from these salts by means of strong organic bases like pyridine. For example,



(b) Alkylation : In basic solution the amino group of an amino acid is nucleophilic and can displace the halogen of alkyl halides.



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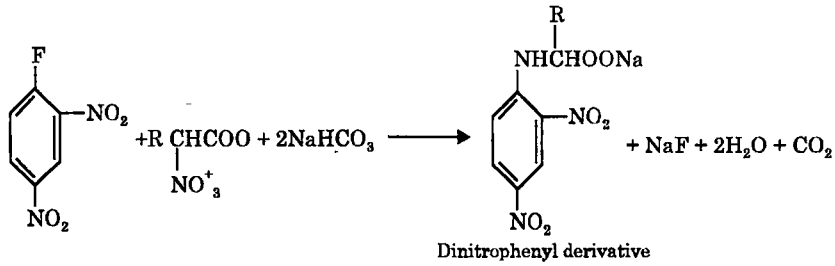
(c) Arylation : In basic solution, the amino group of an amino acid is nucleophilic and can displace the halogen of arylhalides. For example, the flourine of 2, 4-dinitrofluorobenzene can be displaced by nucleophilic agents. The strong electron-withdrawing nitro groups *ortho* and *para* to the fluorine stabilize the intermediate carbanion formed during the displacement. The reaction is generally carried out in mildly alkaline solution.



Dinitrophenyl derivative

The dinitrophenyl derivatives are nicely crystalline compounds. This reaction is quite useful in determining which amino acid in a polypeptide has a free amino group.

(d) With acylating and benzoylating reagents : With acetyl chloride or acetic anhydride, amino acids may be acetylated.

$$R{-}\underset{\displaystyle NH_3^+}{\underset{|}{C}}HCOO^- + (CH_3CO)_2O \longrightarrow RCH\,(NHCOCH)_3\,COOH + CH_3COOH$$

The acetylated amino acid does not exist in a dipolar form and, in general, behaves as a typical organic acid.

Similarly, with benzoyl chloride, amino acids yield benzoyl derivatives.

(e) With nitrous acid : Nitrous acid converts amino acids into hydroxy acids with retention of the configuration. Measurement of the nitrogen evolved is the basis of Van slyke estimation of amino acids.

$$R{-}\underset{\displaystyle NH_3^+}{\underset{|}{C}}H{-}COO^- + HNO_2 \longrightarrow R{-}\underset{\displaystyle OH}{\underset{|}{C}}H{-}COOH + N_2 + H_2O$$

The amino acids proline and hydroxyproline do not react, and the amino group of lysine reacts, but at a slower rate.

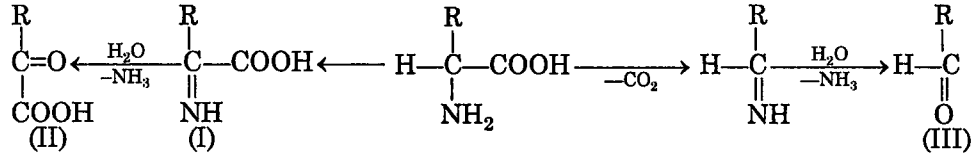
(f) With nitrosyl chloride or bromide : Amino acids react with these reagents to form chloro or bromo acids.

$$RCH(NH_2)COOH + NOCl \longrightarrow RCHCl\,COOH + N_2 + H_2O$$
$$RCH(NH_2)COOH + NOBr \longrightarrow RCHBr\,COOH + N_2 + H_2O$$

(g) With hydriodic acid : When amino acids are heated with hydriodic acid at 200°C, the amino group is eliminated in these amino acids to form fatty acids.

$$RCH(NH_2)COOH \xrightarrow[200°C]{HI} RCH_2COOH + NH_3$$

(h) With oxidising agents : The oxidising agents attack free amino acids at the amino group. Initially either an α-amino acid (I) is formed which subsequently will be hydrolysed to an α-keto acid (II) and ammonia or the oxidation is accompanied by decarboxylation giving rise to the final production of the next lower aldehyde (III) and ammonia.



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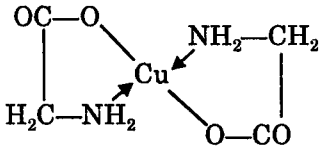
4. Reaction due to the Carboxylic group

(a) With alkalis : Amino acids react with alkali to form salts.

$$H_3^+NCH_2COO^- + NaOH \longrightarrow H_2NCH_2COONa + H_2O$$

(b) With salts of heavy metals : Amino acids form chelate compounds with the salts of the heavy metals. For example, the copper salt of glycine (deep blue needles) is formed by heating copper oxide with an aqueous solution of glycine.



(c) With alcohols : Amino acids readily undergo acid-catalysted esterification if a strong acid is present in excess of the amount required to protonate the amino group. In order to prepare methyl or ethyl ester suspension of amino acid in excess alcohol is made saturated with anhydrous hydrogen chloride. Upon evaporation of the alcohol and excess hydrogen chloride from the solution that results, the ester hydrochloride is formed.

$$\underset{\underset{NH_3^+}{|}}{RCH—COOH} + R'OH \text{ (Excess)} \overset{H^+}{\rightleftharpoons} \underset{\underset{NH_3^+}{|}}{RCHCOOR'} + H_2O$$

(d) With reducing agents : Amino acids can be reduced to the corresponding amino alcohols by reduction with lithium aluminium hydride.

$$\underset{\underset{NH_3^+}{|}}{R—CH—COO^-} \overset{LiAlH_4}{\rightleftharpoons} \underset{\underset{NH_3^+}{|}}{R—CH—CH_2OH}$$

(e) Formation of acid chlorides : In the formation of amino acid chlorides, the amino group is first *"protected"* by treating it with acetic anhydride. The acetylated amino acid formed is converted to the acetylated amino acid chloride with phosphorous pentachloride.

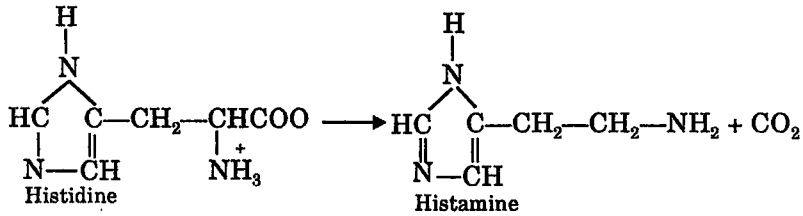
$$\underset{\underset{NH_3}{|}}{R—CHCOO^-} + (CH_3CO)_2O \xrightarrow[-CH_3COOH]{} \underset{\underset{NHCOCH_3}{|}}{RCHCOOH} \xrightarrow{PCl_5} \underset{\underset{NHCOCH_3}{|}}{RCH(C=O)Cl} + POCl_2 + HCl$$

The amino group must be kept in the form of acetyl derivative to prevent reaction between it and the acid chloride group to form a peptide bond.

(f) Decarboxylation : Amino acids may be decarboxylated by heat, acids, bases, barium oxide or specific enzymes to yield the corresponding amine.

$$\underset{\underset{NH_2}{|}}{R—CH—COOH} \longrightarrow \underset{\underset{NH_2}{|}}{R—CH_2—CO_2}$$

For example, decarboxylation of the amino acid histidine yields histamine.



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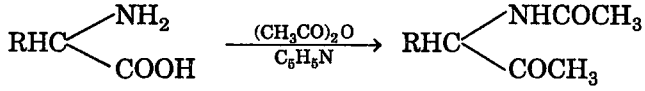
Histamine stimulates the flow of gastric juice into the stomach and is involved in allergic responses.

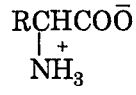
Certain bacteria possess amino acid decarboxylates which convert amino acids in the animal body to amines. Two amino compounds, which are found in the decaying flesh, have names that are very descriptive of their occurrence and their odour : *cadaverine,* $H_2N(CH_2)_5NH_2$ which is produced from the decarboxylation of lysine, and *purtrescine,* $H_2N(CH_2)_4NH_2$ which arises from the decarboxylation of ornithine (2, 5d-diamino pentanoic acid).

(g) Dakin-west reaction : When amino acids are heated with acetic anhydride in pyridine solution, these are converted into methyl α-acetamidoketones. This reaction is often referred to as the Dakin-West reaction.



5. Reactions ue to Both the Amino and Caroxyl Groups

(a) Dipolar ions : When the diopole moment of glycine (and other amino acids) is measured in aqueous solution, this value is found to be very large. In order to explain this large value of dipolemoment it is argued that glycine (and other amino acids) exists as *inner salt* in solution. Such a double charged ion is also known as a *zwitterion, ampholyte* or *a diplar ion.* This may be thought to be produced by the reaction of the acidic carboxylic group with the basic amino group.



Several evidences have been cited to favour the existence of the dipolar ion both in the crystal and in aqueous solution. Some of these evidences are as follows :

(*i*) Spectroscopic studies reveal the absence of the characteristic vibration frequencies for the free carboxyl and the free amino group.

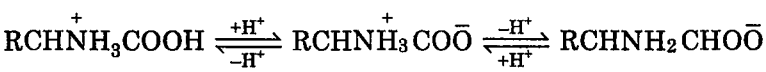
(*ii*) The dielectric constants of solutions of amino acids are very high. This could easily be accounted for by the presence of the dipolar ionic form.

(*iii*) The melting points of amino acids are much higher than the corresponding other substituted acids. The high melting point of the amino acids is indicative of large intermolecular forces in the crystal that arise from the electrostatic attraction of the dipolar ions for each other. On the other hand, the substituted compounds other than the amino acids cannot exist in dipolar form and have relatively low melting points.

(*iv*) Amino acids have low solubility in non-polar solvents. This is expected for dipolar molecules.

The weight of evidence given as above forces us to accept the dipolar ionic form of amino acids.

(b) Isoelectric point : In neutral solution, the following equilibrium exists in the following species :



Conjugate acid Conjugate base

The prodominant form in which the amino acids exist in solution is dependent upon the pH of the solution. Thus, is acid as added, the equilibrium will shift in the favour of the cation; as base is added, the equilibrium will shift in the favour of the anion. For each amino acid, there is

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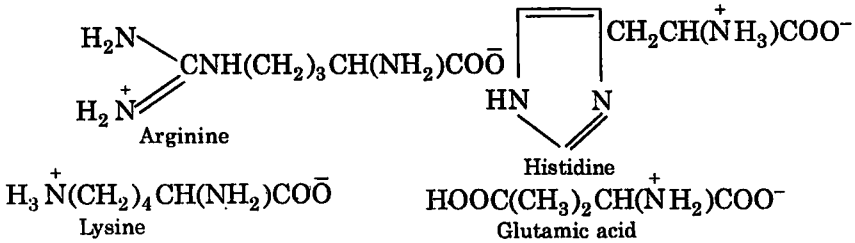
a particular pH at which the concentrations of the anion and cation in the solution will be equal at a minimum, and the concentration of the dipolar ion in the solution will be at a maximum. Since the dipolar ion has a net charge of zero, the migration of ions under the influence of an electric field is called the isoelectric point of the amino acid. The isoelectric points for the various amino acids are given in Table 7.2. Since for monoaminocarboxylic acids the isoelectric point is attained by adding hydrogen ion to the solution, isoelectric point for a monoaminomonocarboxylic acid will be on the acid side of pH 7. For example, the isoelectric point of glycine is pH 5.9.

If there are two amino to two carboxyl groups in an amino acid, several structures are possible for the dipolar ion of amino acid at the isoelectric point. It is to be remembered that ionisation of α-carboxyl group is involved in all α-amino acids.



(c) Sorensen formal titration : The monoaminomonocarboxylic acids have two pK values because these behave both as an acid and as a base. By convention, pK_1 corresponds to the group titrated at the most acid region, *i.e.,* the carboxylic group. Titration of an amino acid with alkali determines the pK_2 of that acid, *i.e.,* the group with the higher pK value is the positively charged NH_3^+ group.

When carboxyl group is to be titrated with alkali, the amino group should be masked. Thus, a formalin solution when added to glycine yields methyleneglycine.

$$H_2NCH_2COOH + HCHO \longrightarrow CH_2 = NCH_2COOH + H_2O$$

Previously, it was thought that methylene-glycine is only formed. But now it is well established that the main product may be dimethyloglycine.

$$H_2NCH_2COOH + 2HCHO \longrightarrow (CH_2OH)_2NCH_2COOH$$

The reaction is used for estimation of amino acids by direct titration with alkali. This method is known as *Sorensen's Formol Titration Method.* The direct titration of amino acid with alkali cannot be carried out because of the presence of free $-NH_2$ group. Reaction with formaldehyde converts amino acids to methylene amino acid, which is a strong acid and can be titrated directly with an alkali.

Table 7.2

Acid	*Isoelectric Point*	*Acid*	*Isoelectric Point*
Glycine	6.0	Methionine	5.7
Alanine	6.1	Tryptophan	5.9
Valine	6.0	Proline	6.3
Leucine	6.0	Hydroxyproline	5.8
Isoleucine	6.0	Aspartic acid	3.0
Phenylalanine	5.9	Asparagine	5.4
Tyrosine	5.6	Glumatic acid	3.1
Serine	5.7	Glutamine	5.7
Cysteine	5.1	Arginine	10.8
Cystine	5.0	Lysine	9.5
Threonine	5.7	Histidine	7.6

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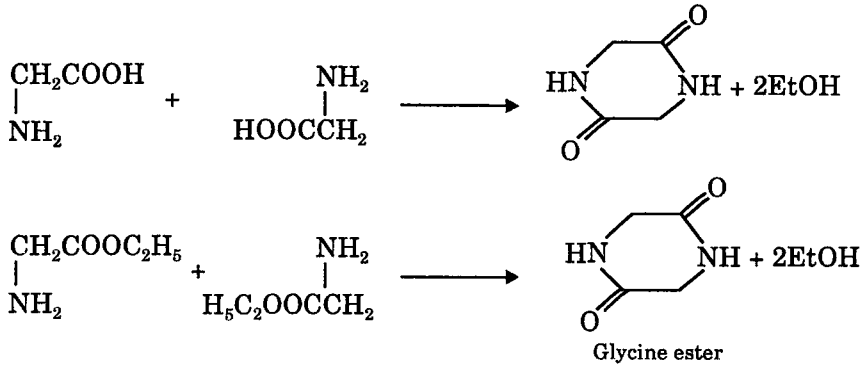
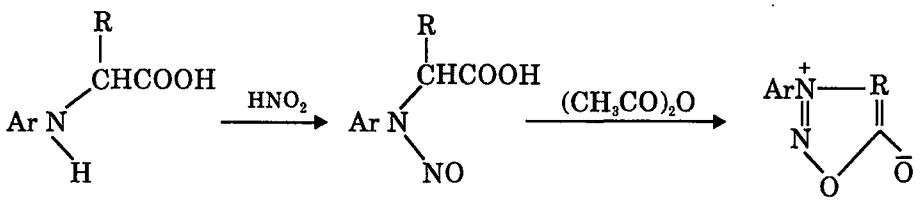
(d) Action of heat : When amino acids are heated, 2, 5 diketopiperazines are obtained; esters give better yields.



Glycine ester

Diketopiperazines are often found as products in the hydrolysis of proteins.

(e) Action of nitrous acid : With nitrous acid, N-alkyl or arylamino acids yield N-nitroso derivatives which upon dehydration with acetic anhydride yield sydnones.



(f) Formation of betaines : The betaines are trialkyl derivatives of the amino acids, these may be prepared by heating amino acids with methyl iodide in methanolic solution. The betaines exist as dipolar ions. For example, the formation of betaine from glycine is written as follows :

$$H_3\overset{+}{N}\,CH_2CO\bar{O} + 3CH_3I \longrightarrow (CH_3)_3\overset{+}{N}CH_2CO\bar{O} + 3HI$$

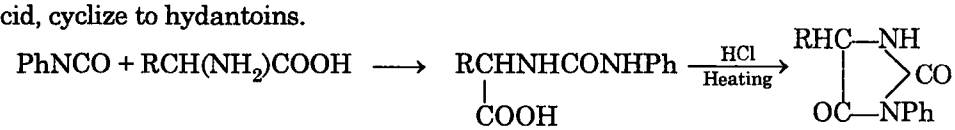
Betaine is more conveniently prepared by warming an aqueous solution of chloroacetic acid with trimethylamine.

$$(CH_3)_3N + ClCH_2COOH \longrightarrow (CH_3)_3\overset{+}{N}CH_2CO\bar{O} + HCl$$

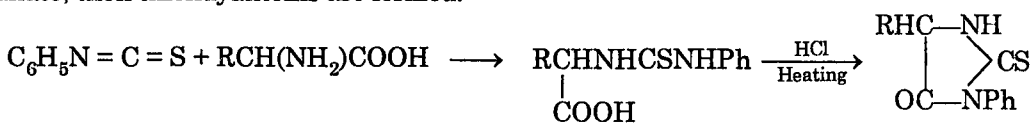
Betaine is a base it, therefore, reacts with hydrochloric acid to form the stable crystalline hydrochloride.

$$(CH)_3\overset{+}{N}CH_2CO\bar{O} + HCl \longrightarrow Cl\bar{}\{(CH)_3\overset{+}{N}CH_2COOH$$

(g) Action of phenyl isocyanate : Like other amino compounds, α-amino acids react with isocyanates to form phehylhydantoic acids. The latter substances on heating, especially in the presence of acid, cyclize to hydantoins.



(h) Action of phenyl isothiocyanate : If phenyl isothiocyanate is used instead of the thiocyanate, then thiohdyantoins are formed.



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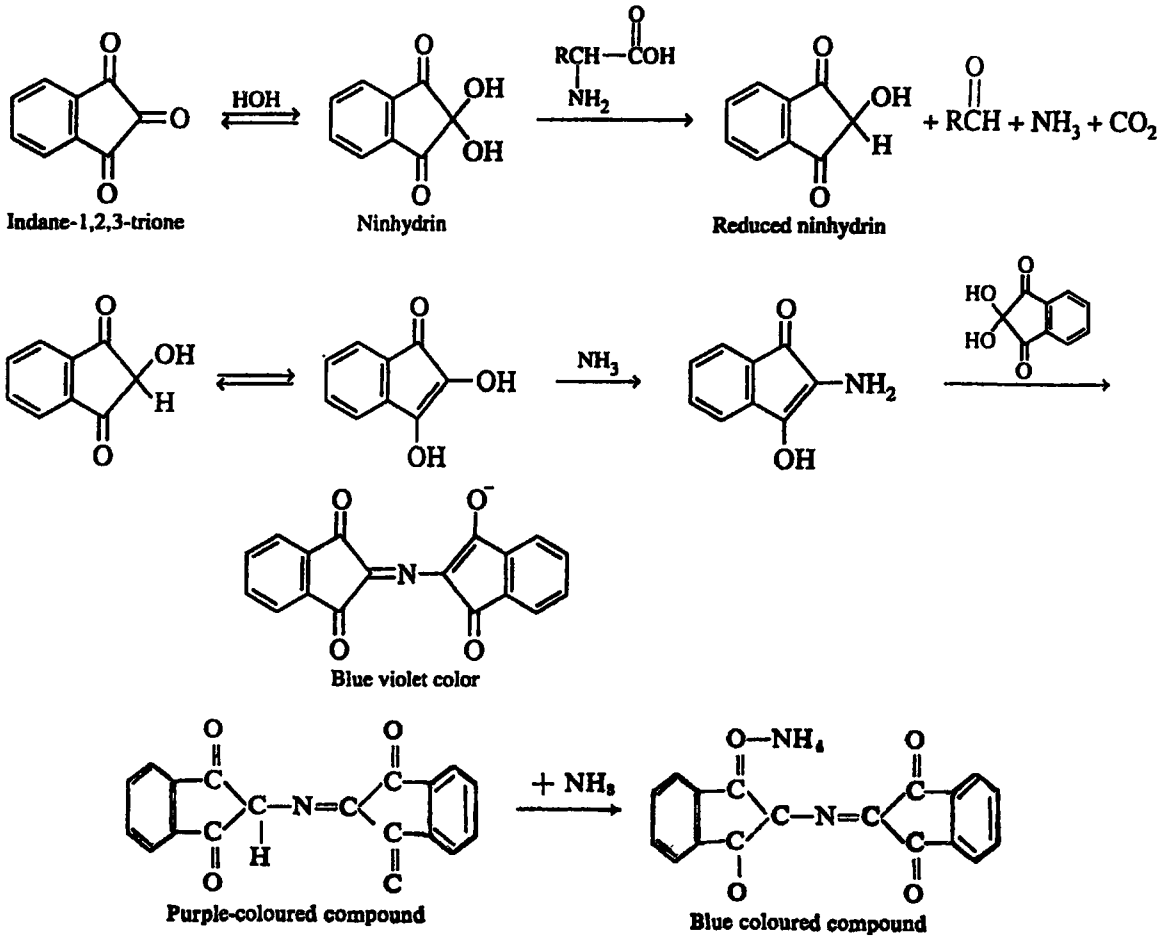
(f) Ninhydrin reaction : With ninhydrin (indane-1, 2, 3 trione hydrate), amino acids yield a coloured product. The mechanism of the reaction is not certain. However, ninhydrin is a powerful oxidising reagent. It brings about oxidative decarboxylation of α-amino acids, producing CO_2, NH_3 and an aldehyde with one less carbon atom than the parent amino acid. The reduced ninhydrin then reacts with the liberated ammonia, forming a blue complex which maximally absorbs light of wavelength 570 nm. The intensity of blue colour produced under standard conditions forms the basis of an extremely useful quantitative test for α-amino acids. Amines other than α-amino acids also react with ninhydrin, forming a blue colour but without evolving CO_2. The evolution of CO_2 is thus indicative of an α-amino acid.



The intense blue product is generally characteristics of those amino acids which are having α-amino acids. However, proline and hydroxyproline, the secondary amines, yield yellow products, and asparagine, which has a free amide group, reacts with ninhydrin to yield a characteristic brown product. The ninhydrin reaction is generally employed to do quantitative estimation of amino acids.

(i) Ultraviolet absorption spectrum of aromatic amino acids : The aromatic amino acids tryptophan, tyrosine, histidine, and phenylalanine absorb ultraviolet light. As shown in Fig. 7.2 most of the ultraviolet absorption of proteins is due to their tryptophan content.

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Electrophoresis

Electrophoresis is a method used for the separation and analysis of amino acids. The method is based on pH control and electric charge. The amino acids differ in their isoelectric points. The mixture of amino acids is placed on the centre of a paper strip (cellulose acetate) at certain pH. The pH is maintained by saturating the paper strip with the buffer solution. The paper strip is attached to two electrodes. On passing electric current through the strip, amino acids migrate towards electrodes depending upon the net charge present on them. For example, a mixture of lysine, alanine, and glutamic acid at pH = 6.0, when placed on a paper strip, on passing current results in the movement of glutamic acid towards anode and of lysine towards cathode. The alanine has no net charge and therefore does not move (Fig. 7.3). The amino acid with isoelectric point greater than the buffer pH gains a proton and becomes positively charged (Lysine, pI = 9.7) and thus moves towards anode. On the other hand, the amino acid with isoelectric point lower than the buffer pH looses a proton and becomes negatively charged (Glutamic acid, pI = 3.2) and thus, moves towards anode. The amino acid with isoelectric point comparable to buffer pH does not migrate towards any electrode, such as Alanine, pI = 6.0.

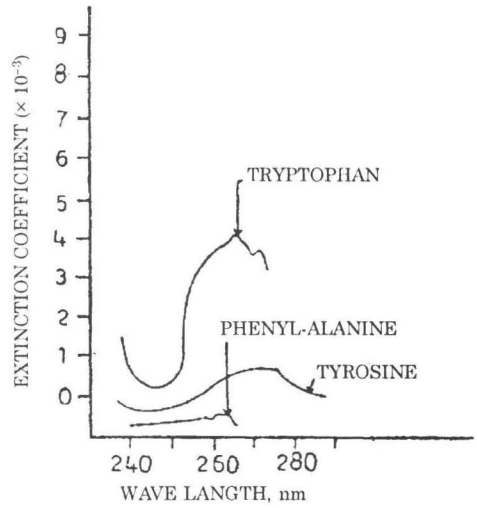

Fig. 7.2. The ultraviolet absorption spectra of tryptophan, tyrosine and phenylalanine.

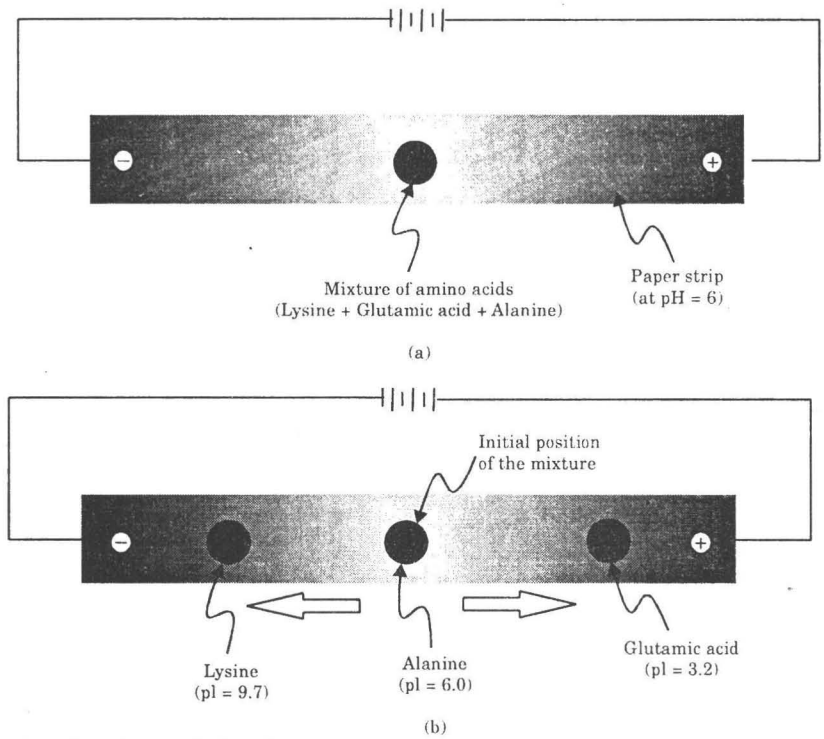

Fig. 7.3. Separation of amino acids by electrophoresis. (*a*) At pH = 6.0, Glutamic acid is negatively charged and moves towards anode. Lysine at this pH is positively charged and moves towards cathode. Alanine remains at the same position, as at pH = 6.0 it has no net charge (*b*) Separation of mixture of amino acids after electrophoresis.

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8 Peptides and Proteins

8.1 Peptides

The interaction of amino group of one amino acid with carboxylic acid group of another amino acid results in the formation of an amide linkage. A number of amino acid units are joined with each other through $-\overset{\overset{O}{\|}}{C}-NH-$ linkages, also known as *peptide linkage*. The peptide with two amino acids is termed as *dipeptide*. Similarly, the amino acid with three amino acid units is called a *tripeptide* and four amino acids *tetrapeptide* and so on.

The peptide bond is a chemical, covalent bond formed between the α-amino group of one amino acid and the α-carboxyl group of another [Fig. 8.1 (*a*)]. Once two amino acids are joined together via a peptide bond to form a dipeptide, there is still a free amino group at one end and a free carboxyl group at the other, each of which can in turn be linked to further amino acids. Thus, long, unbranched chains of amino acids can be linked together by peptide bonds to form oligopeptides (up to 25 amino acid residues) and polypeptides (> 25 amino acid residues).

The peptide bond between the carbon and nitrogen exhibits *partial double-bond character* due to the closeness of the carbonyl carbon-oxygen double-bond allowing the *resonance structures* in [Fig. 8.1 (*b*)] to exist. Because of this, the C—N bond length is also shorter than normal C—N single bonds. The *peptide unit* which is made up of the CO—NH atoms is thus relatively rigid and planar, although free rotation can take place about the C_α—N and C_α—C bonds (the bonds

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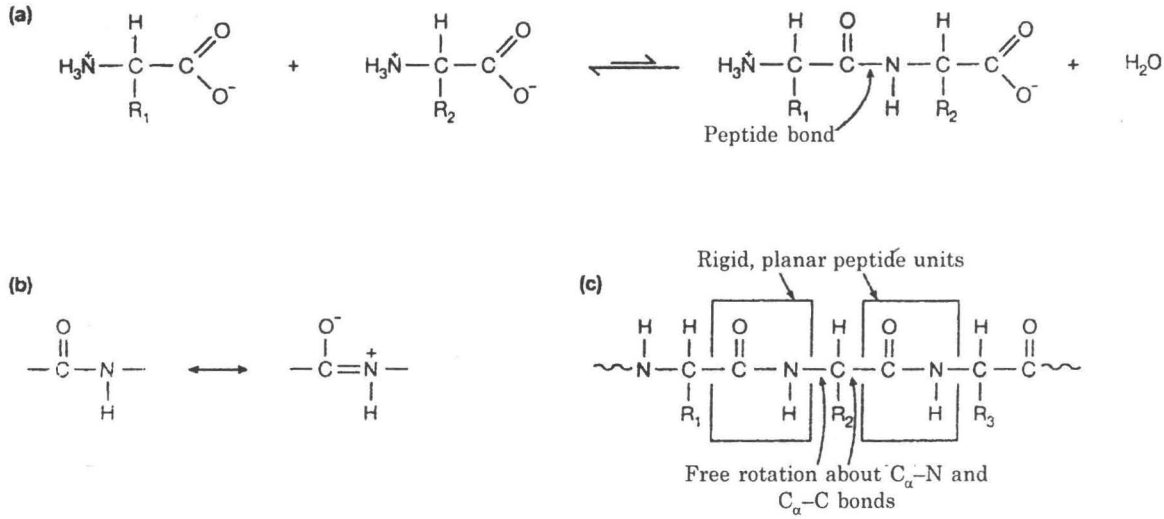

Fig. 8.1. (*a*) Formation of a peptide bond, (*b*) resonance structures of the peptide bond, (*c*) peptide units within a polypeptide.

on either side of the peptide bond), permitting adjacent peptide units to be at different angles [Fig. 8.1 (*c*)]. The hydrogen of the amino group is nearly always on the opposite side (*trans*) of the double bond to the oxygen of the carbonyl group, rather than on the same side (*cis*).

The blackbone of a polypeptide is a linked sequence of rigid planar peptide groups. The backbone conformation of a polypeptide is specified by the *rotation angles* or *torsion angles* about the C_α–N bond (*phi*, ϕ) and C_α–C bond (*psi*, ψ) of each of its amino acid residues. When the polypeptide chain is in its planar, fully extended (all-*trans*) conformation the ϕ and ψ angles are both defined as 180°, and increase for a clockwise rotation when viewed from C_α (Fig. 8.2). The *conformational range* of the torsion angles, ϕ and ψ, in a polypeptide backbone are restricted by steric hindrance. The sterically allowed values of ϕ and ψ can be determined by calculating the distances between the atoms of a tripeptide at all values of ϕ and ψ for the central peptide unit. These values are visualized in a steric contour diagram, otherwise known as a conformation map or *Ramachandran plot* (Fig. 8.3). From Fig. 8.3 it can be seen that most areas of the Ramachandran plot (most combinations of ϕ and ψ) are conformationally inaccessible to a polypeptide chain. Only three small regions of the conformation map are physically accessible to a polypeptide chain, and within these regions are the ϕ–ψ values that produce the right-handed α-helix, the parallel and antiparallel β-pleated sheets and the collagen helix.

The polypeptide chain folds up to form a specific shape *(conformation)* in the protein. This conformation is the *three-dimensional arrangement* of atoms in the structure and is determined by the amino acid sequence. There are four levels of structure in proteins : *primary, secondary, tertiary* and, sometimes but not always, *quaternary.*

In accordance with the number of amino acid units present, peptides may be classified as follows :

1. Oligopeptides (2–25 amino acids)
2. Polypeptides (25–100 amino acids)
3. Proteins (> 100 amino acids)

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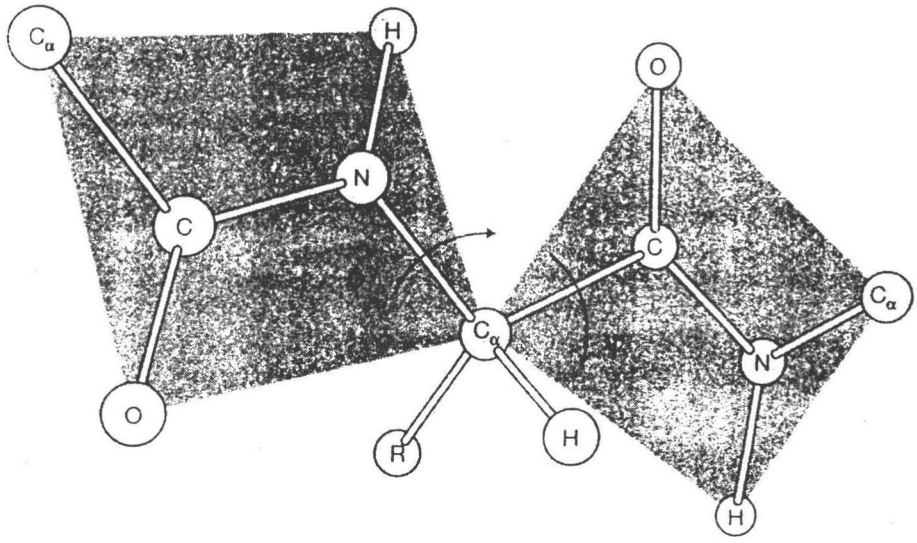

Fig. 8.2. A segment of a polypeptide chain showing the torsion angles about the C_α—N bond (ϕ) and C_α–C bond (ψ).

8.2 Synthesis of Polypeptides

Suppose we consider the synthesis of peptides by taking example of combination of glycine and valine (without repetition of either amino acid) which can give rise to the formation of two different dipeptides.

(*a*) Carboxylic group of glycine reacting with amino group of valine, that is, amino group of glycine (as *N*-terminal) is free and carboxylic group (as *C*-terminal) of valine is free. The dipeptide thus formed is known as *Glycylvaline.*

(*b*) Carboxylic group of valine reacting with amino group of glycine, that is, amino group of valine (as *N*-terminal) is free and carboxylic group (as *C*-terminal) of glycine is free. The dipeptide thus formed is known as *Valylglycine.*

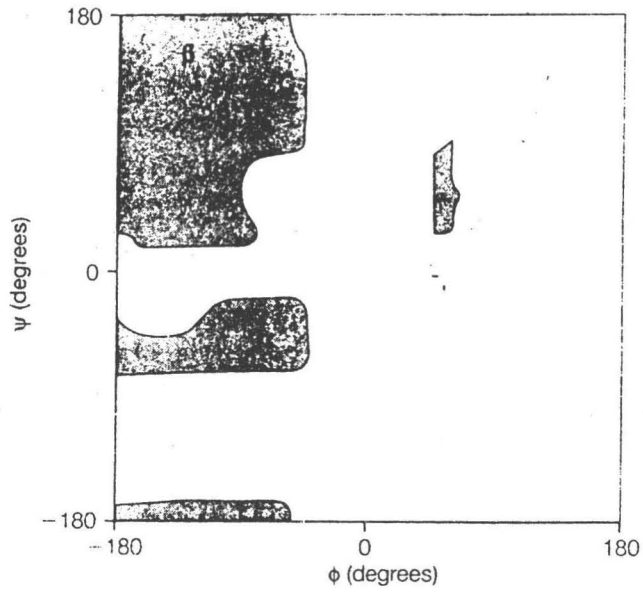

Fig. 8.3. Ramachandran plot showing the allowed angles for poly-*L*-alanine (grey regions). α, ϕ–ψ values that produce the right-handed α-helix; β, the antiparallel β-pleated sheet; β′, the parallel β-pleated sheet; *C*, the collagen helix.

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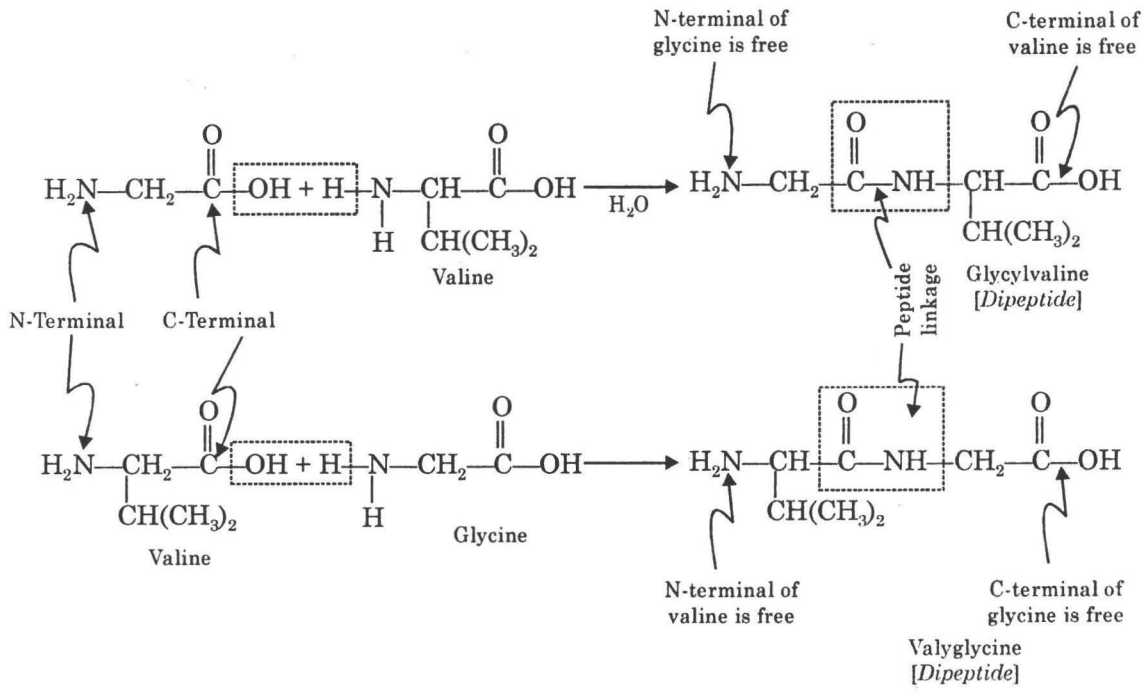
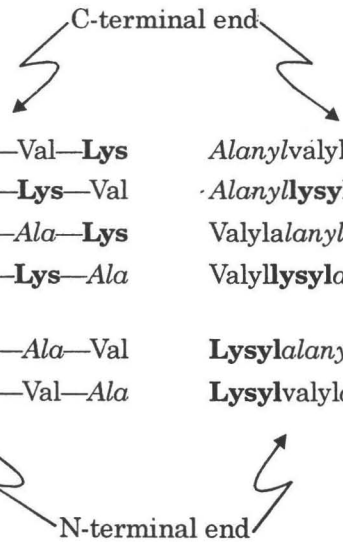


In an analogous manner, *three* different amino acids—alanine (Ala), valine (Val), and leucine (Lys) can form *six* different tripeptides (without repetition of any amino acid), which are given as follows :



(*i*) *Ala*—Val—**Lys**	*Alanyl*valyl**lysine**
(*ii*) *Ala*—**Lys**—Val	*Alanyl***lysyl**valine
(*iii*) Val—*Ala*—**Lys**	Valyl*alanyl***lysine**
(*iv*) Val—**Lys**—*Ala*	Valyl**lysyl***alanine*
(*v*) **Lys**—*Ala*—Val	**Lysyl***alanyl*valine
(*vi*) **Lys**—Val—*Ala*	**Lysyl**valyl*alanine*

Six possible combinations of tripeptides from three different amino acids. [Other possibilities of the formation of several peptides by the combination of same amino acid twice or thrice (and so on...) and other peptides with more than three linkages is not mentioned here.]

With an increase in the number of amino acids, the possible combinations of polypeptides also increase tremendously. If an amino acid is used only once, 20 different amino acids would be having 2.4×10^{18} combinations. Nature, in fact, uses these 20 amino acids as building blocks to create variety of proteins, which carry out diversified functions.

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8.3 Use of Protecting Groups in Synthesis of Polypeptides

In the synthesis of peptides, precaution has to be taken to prevent the interaction of amino group of an amino acid with carboxylic group of the same amino acid. For example, when dipeptide *glycylvaline* is synthesized from *glycine* and *valine* amino acids, the following possibilities exist :

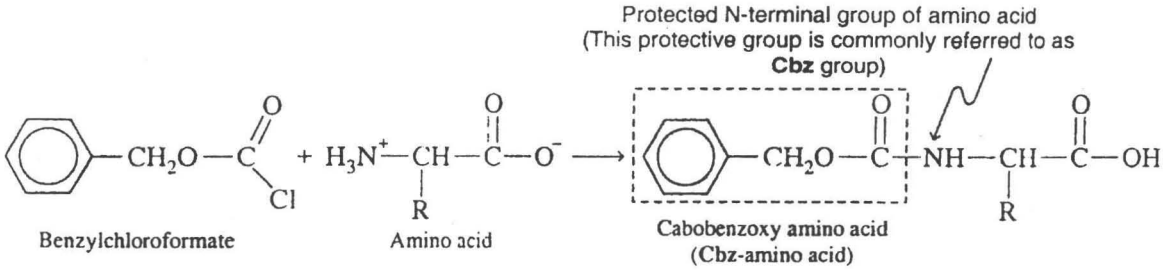
(Glycine + Valine) ⇒ **Glycylvaline** } Required peptide
Valylglycine
Glycylglycine } *Other possible dipeptides*
Valylvaline *(Side products)*

To synthesize glycylvaline only, the $—NH_2$ group of glycine and —COOH group of valine must be protected so that they are not freely available to react at these junctions. This is described in the following sections :

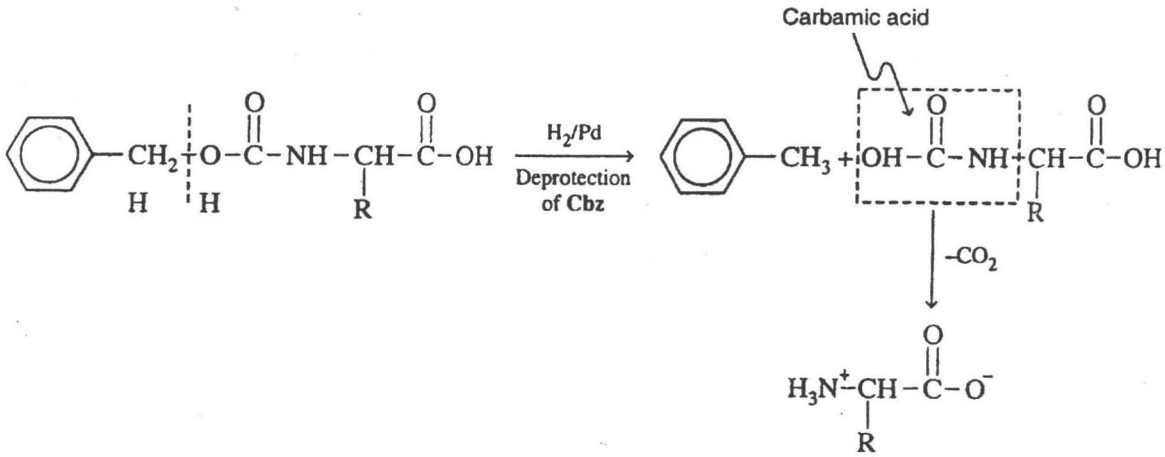
Protection of Amino Group ($—NH_2$)

The amino end is protected or blocked by employing the following groups :

(*i*) *Phenylmethoxycarbonyl group (Carbobenzoxy; Cbz) :* This group is in abbreviated as *Cbz* group. The carbobenzoxy group is introduced into amino acid by reacting an amino acid with *phenylmethyl chloroformate* (*benzylchloroformate*).



The deprotection of *Cbz group* is done by hydrogenolysis in the presence of palladium (H_2/Pd), which first forms a carbamic acid that readily undergoes decarboxylation to yield free amino group.



(*ii*) *1, 1-Dimethylethoxycarbonyl group (tert-butoxycarbonyl; BoC).* This group is abbreviated as *BoC*. It is introduced into amino acid (for blocking the amino group) by reacting amino acid with bis-1, 1-dimethylethyl dicarbonate (di-*tert*-butyldicarbonate).

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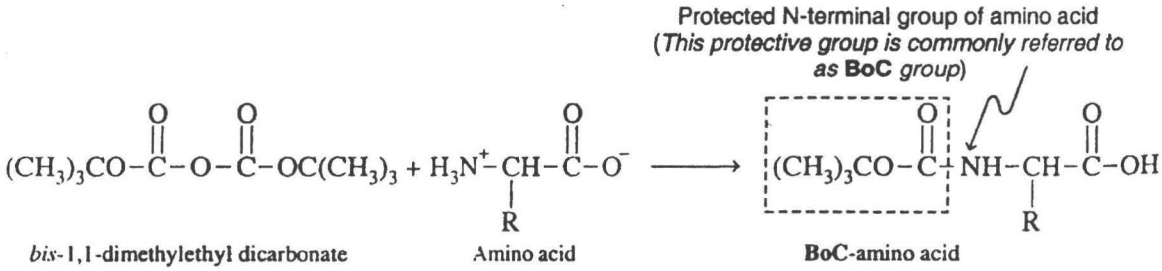
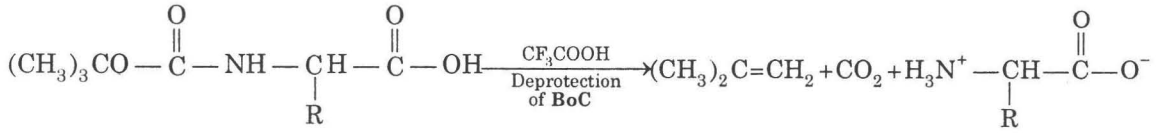
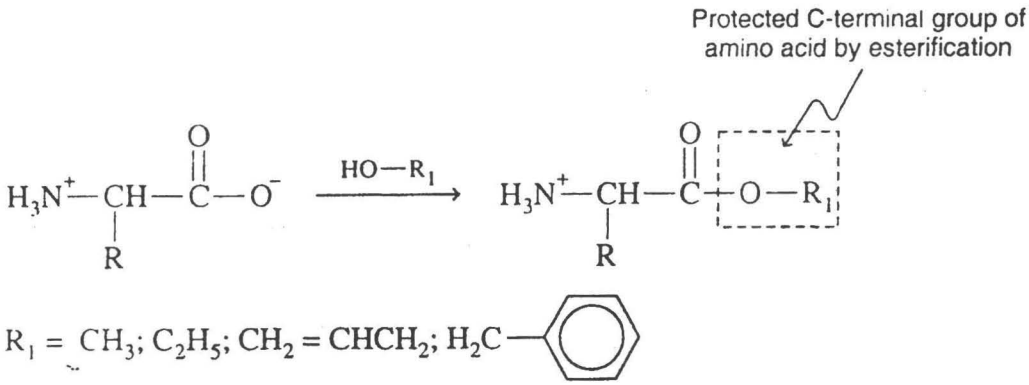


The reverse process or deprotection of tert-butoxycarbonyl group (BoC) is performed by treating the protected amino acid with hydrogen bromide in the presence of acetic acid or by reacting it with trifluoroaceticacid (CF_3COOH).



Protection of Carboxylic Group (—COOH)

Ester groups, for example methyl, ethyl, allyl, or benzyl ester are able to protect the carboxylic end of amino acid.



Reacting the protected amino acid with base carries out deprotection at the carboxylic group.

8.4 Synthesis of Peptides Using Protected Amino and Carboxylic Acid Ends

Formation of Peptide Linkage : Use of DCC

Once amino group and carboxylic group of amino acids are protected, the peptide formation is done by converting carboxylic group of N-terminal protected amino acid to its acyl derivative. Many such methods are available which bring about the activation of carboxy group. The most important and widely used method involves *DCC (dicyclohexylcarbodiimide).* DCC activates the carboxylic carbon so that the nucleophilic attack of amino group of another amino acid takes place readily at carboxylic carbon. DCC acts as a dehydrating agent.

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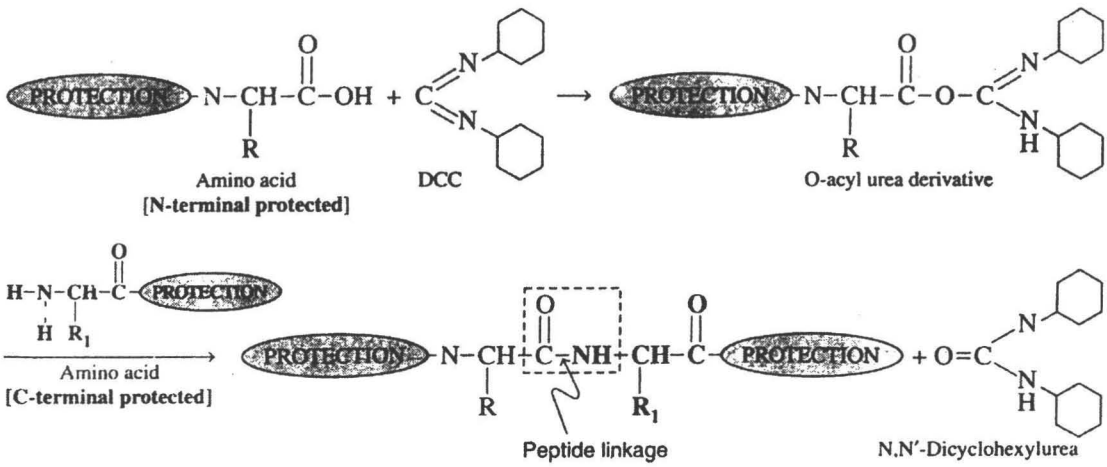
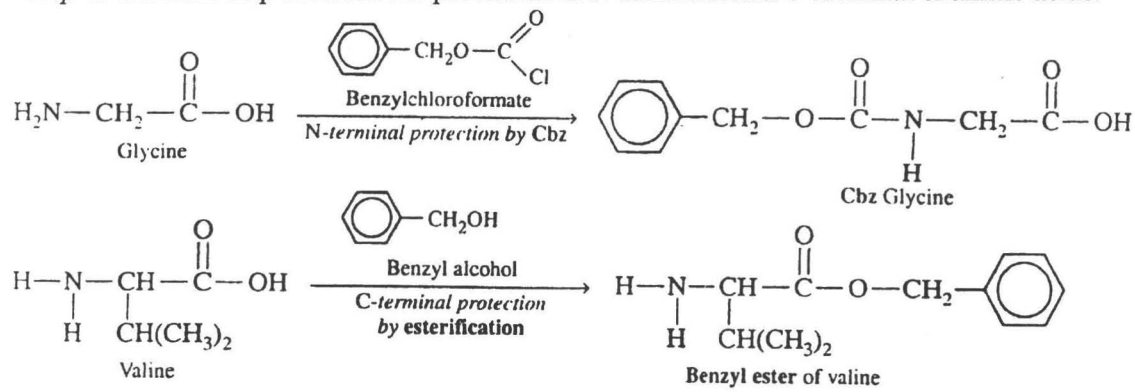
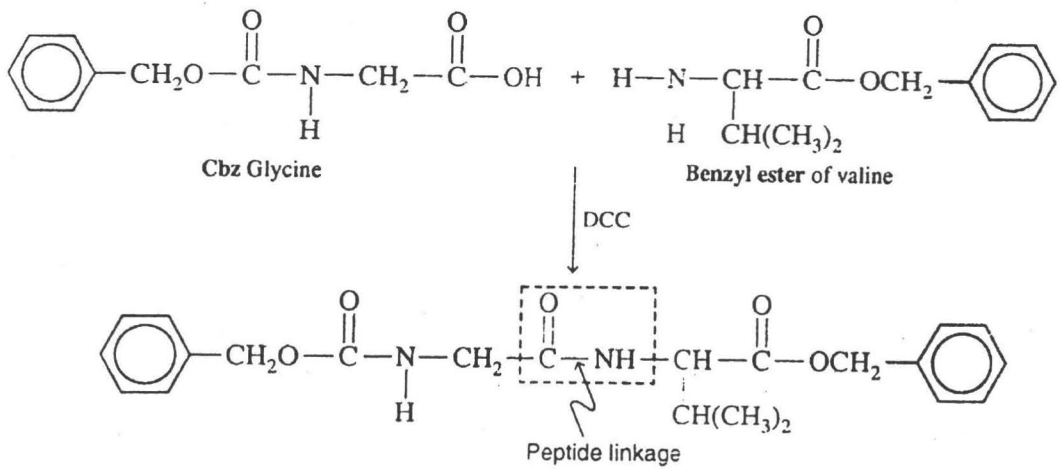


Now peptide chain is formed by carrying out the deprotection. (Same procedure follows in the synthesis of peptides using more number of amino acids). Thus, we can describe the synthesis of dipeptide glycylvaline (gly-val) in the following steps :

Step 1. The first step involves the protection of N-terminal and C-terminal of amino acids.



Step 2. The second step involves the peptide formation in presence of DCC.



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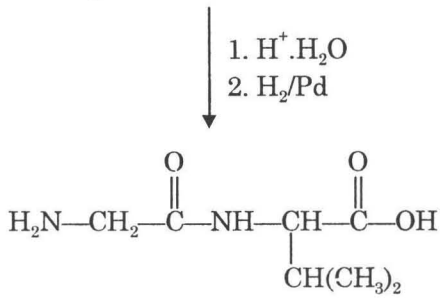
Step 3. The third step involves deprotection of terminals



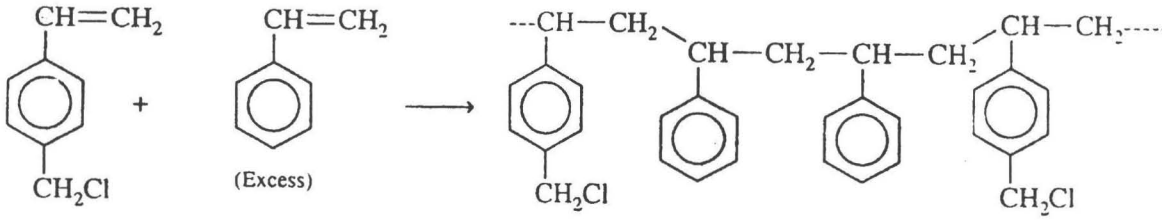
Glycylvaline [*Dipeptide*]
(*Only product formed*)

8.5 Solid Phase Polypeptide Synthesis

[Merrifield solid phase polypeptide synthesis]

The synthesis of polypeptide chain by additing one amino acid at a time, in a sequence, to the growing peptide chain is a time consuming process. This involves the purification of peptide at each and every addition of amino acid. This major hurdle has been overcome by using the technique of solid phase peptide synthesis as developed by Robert B. Merrifield who synthesized Ribonuclease enzyme having 124 amino acids in just six weeks.

The Merrifield method involves the use of a solid support, which consists of beads of a copolymer. The copolymer is synthesized from reaction of styrene with 4–(chloromethyl) styrene.



The synthesis (Fig. 8.4) involves in the following steps :

Step 1. The N-terminal protected (BoC) amino acid has been attached to the copolymer through ester bond formation between —COOH of amino acid and —CH_2Cl of the copolymer.

Step 2. The copolymer attached to amino acid is washed to remove excess reagents. The reaction with trifluoroacetic acid (CH_3COOH) is then carried out to remove BoC protecting group so that a free amino group becomes available.

Step 3. Another amino acid (amino group protected with BoC) is then added to copolymer in the presence of DCC. The free amino group of amino acid attached to polymer forms peptide linkage with free carboxylic group of second amino acid.

Step 4. Copolymer and the attached dipeptide is again washed for removing excess reagent for the addition of another amino acid. Steps 2 and 3 are now repeated. These steps can be repeated a number of times depending upon the length of polypeptide chain.

Step 5. Once the required polypeptide is synthesized by linking of different amino acids the polypeptide is released from the copolymer by reaction with hydrofluoric acid (HF). Hydrofluoric acid is having two functions :

(*a*) It cleaves the ester bond and leaves the peptide chain intact.

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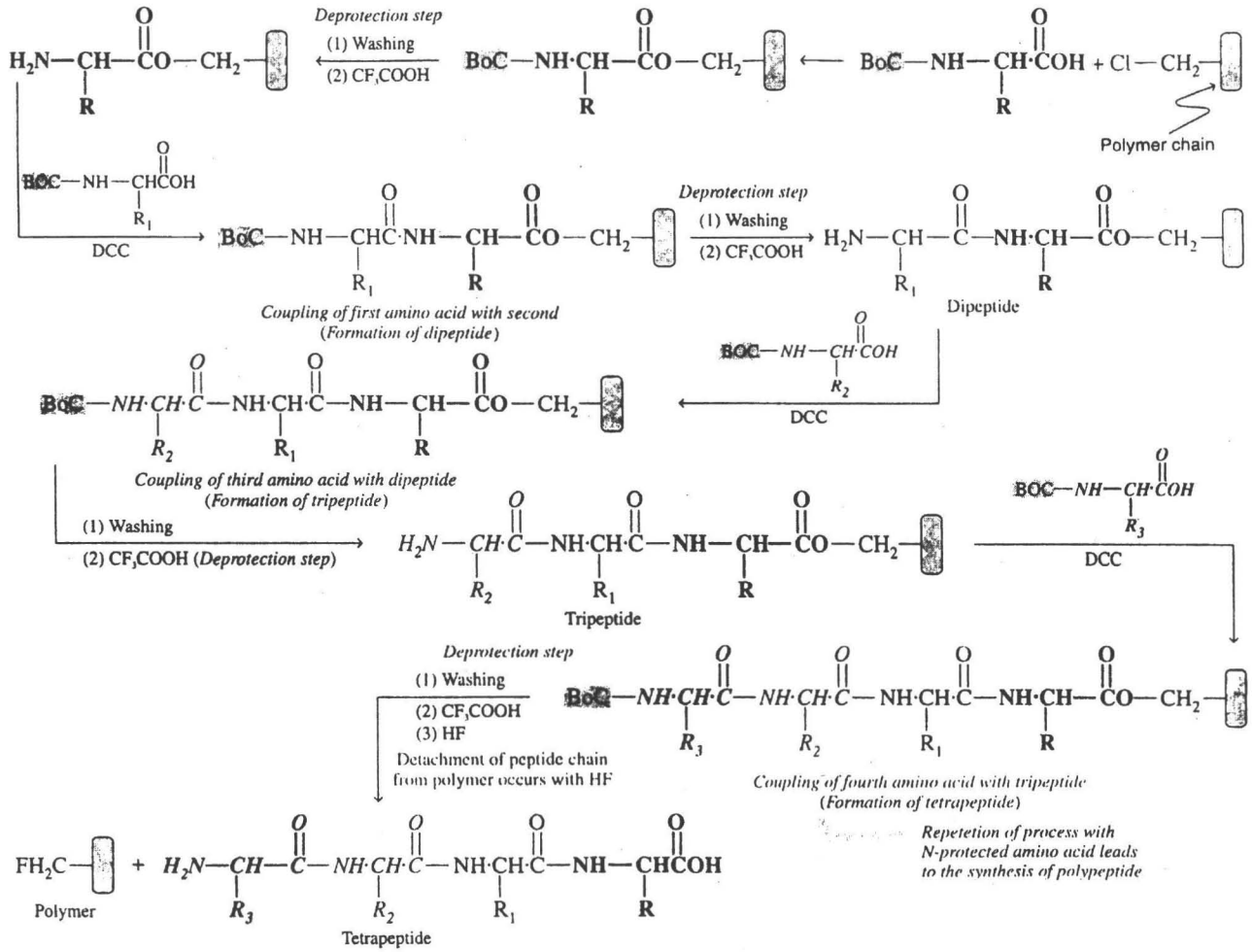

Fig. 8.4. Merrifield solid phase polypeptide synthesis (Schemaic representation for synthesis of tetraptide). The four amino acids used in tetrapeptide synthesis are shown in four different ways as bold, light, italics and italics bold faced to show the distinction.

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(*b*) It removes the N-terminal protecting group, namely BoC.

The Merrifield solid phase peptide synthesis is presently carried out with automated peptide synthesizer which automatically undertakes the washing and removal of protecting group at each addition step.

Sequence Strategy

In order to sequence an entire protein, the polypeptide chain has to be broken down into smaller fragments using either chemicals (*e.g.*, cyanogen bromide) or enzymes (*e.g.*, chymotrypsin and trypsin). The resulting smaller fragments are then sequenced by Edman degradation. The complete sequence is assembled by analyzing overlapping fragments generated by cleaving the polypeptide with different reagents. Aminopeptidase and carboxypeptidase release the N- and C-terminal amino acids from a protein, respectively. The polypeptides in a multi-subunit protein have to be dissociated and separated prior to sequencing using urea or guanidine hydrochloride which disrupt non-covalent interactions, and 2-mercaptoethanol or dithiothreitol that break disulfide bonds.

Determination of Sequence of Amino Acids in a Polypeptide

The most interesting aspect in the study of amino acids is to find out the constituent amino acids in a polypeptide and the sequence in which these are arranged. The determination of sequence of amino acids in a polypeptide involves the following steps :

(1) A polypeptide (protein) is isolated and purified by different techniques, for example, dialysis, gel filtration chromatography, ion-exchange chromatography, electrophoresis, and so on. The electrophoresis is regarded to be highly efficient and powerful technique for separation and purification.

(2) The constituent amino acids of a polypeptide are determined by hydrolysis of polypeptide chain. The entire chain is degraded by hydrolysis in the presence of an acid (6N HCl) and this gives rise to the formation of a mixture of amino acids.

(3) The qualitative and quantitative estimation of amino acids are done with the help of an automated amino acid analyzer. The basic principle involved here is ion-exchange chromatography. In the process, the acidic amino acids are released first and the most basic amino acid are released in the end.

(4) The next step involves the identification of N- and C-terminals, that is, *end group analysis.*

(5) Once the end groups are identified, stepwise degration of peptide chain is done to determine the order of amino acids (sequence) in a chain. The process involves the non-enzymatic hydrolysis of polypeptide into smaller fragments.

The last two steps, namely end group analysis and sequence determination of polypeptides have been now discussed in detail.

End Group Analysis

The N-terminal is having a free amino group and the C-terminal is having a free carboxylic group. Thus, amino acids present at two terminals are completely different from each other as well as from other amino acids present in a chain.

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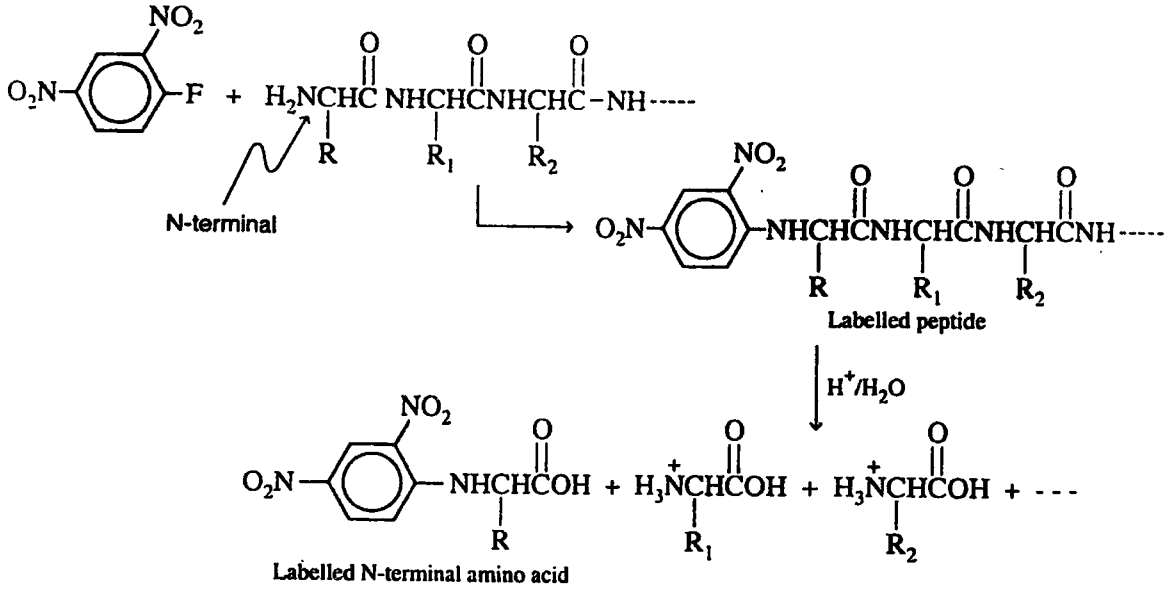
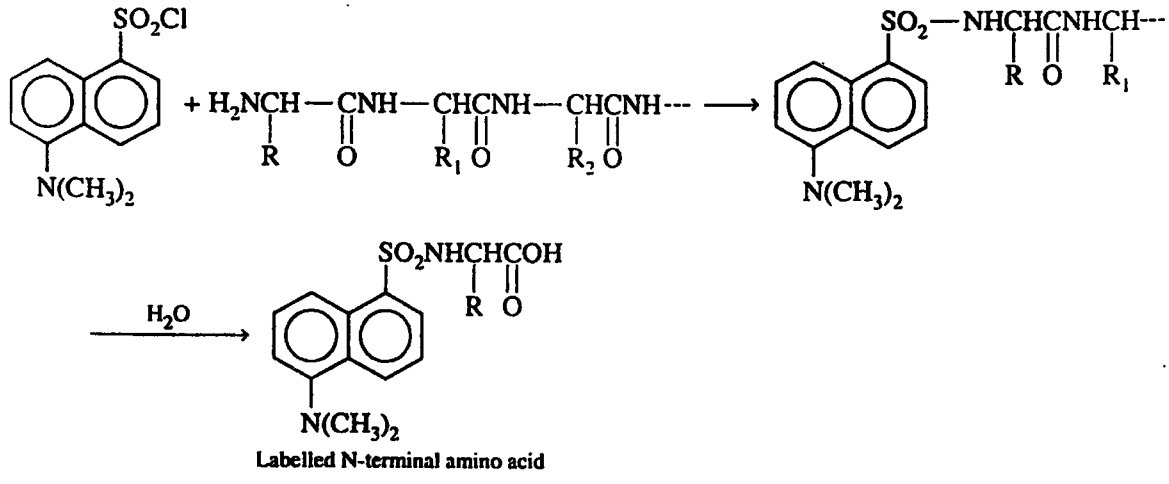
N-terminal Analysis

The N-terminal residue can be identified by the following three methods :

(*i*) *DNP method :* This method was developed by F. Sangar. In this method the peptide chain is treated with 2, 4-dinitrofluorobenzene (DNFB), which brings about substitution of DNFB by free amino group to yield a N-Dinitrophenyl (DNP) derivative. The hydrolysis of peptide chain gives rise to the separation of labelled N-terminal amino acid (DNP-amino acid) and a mixture of another amino acids. Hence, DNP-amino acid so separated has been identified to determine the N-terminal.



(*ii*) *Dansyl method :* In this method the reaction of peptide chain with 5-dimethylaminonaphthalene-1-sulfonylchloride (Dansyl chloride) takes place at the N-terminal (amino end). The hydrolysis of peptide chain gives rise to the formation of Dansyl amino acid. This Dansyl amino acid on irradiation with UV light shows fluorescence. Thus, the N-terminal is identified. This method has been 100 times more sensitive than DNP method in detection of the N-terminal.



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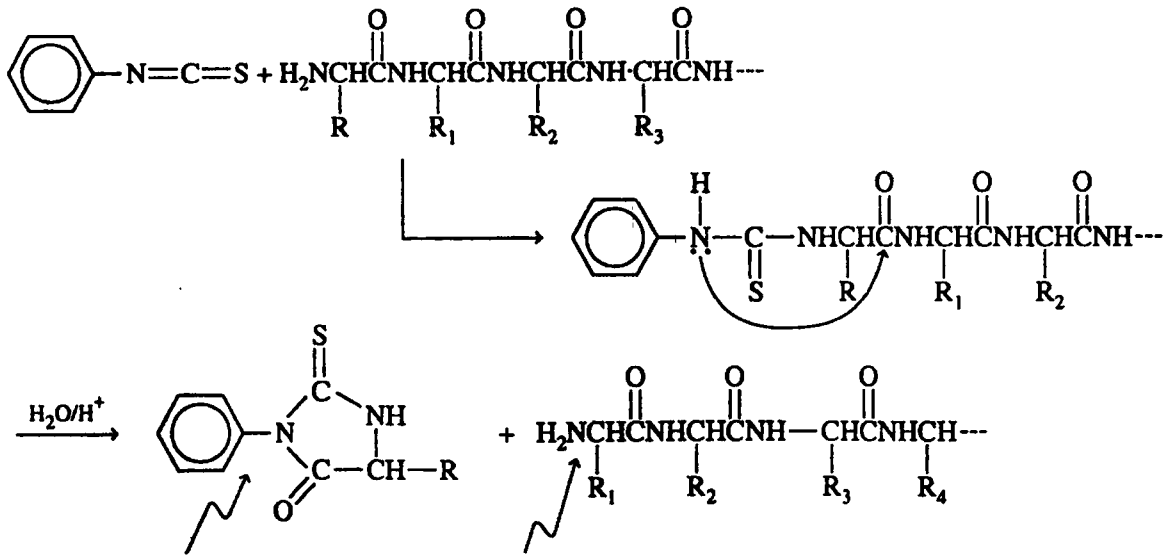
(*iii*) *Edman degradation :* It is one of the most important and widely used method which depends on the selective removal of N-terminal amino acids from polypeptide chain (proteins). The method removes N-terminal amino acid and leaves the rest of the polypeptide chain intact. Hence, the method can be employed repeatedly to identify the N-terminal of shortened peptide chain.

Pehr Edman of Max Planck institute of biochemistry, Munich, developed this method. It involves treatment of polypeptide with phenylisothiocyanate. The N-terminal of the polypeptide undergoes reaction with phenylisothiocyanate to form substituted-thiourea, that on mild hydrolysis yields the selective separation of N-terminal amino acid as phenylthiohydantion, which is identified by HPLC (High Performance Liquid Chromatography). The rest of the polypeptide chain remains intact (of course with one N-terminal amino acid less). The method can be repeated on this new N-terminal and the process may be repeated.

The automation of above process has increased the efficiency of this method to continue sequencing the amino acids in polypeptides. The repeated Edman degradation can be used for identification of polypeptides having 50 or even more amino acids. This method is not applicable for high polypeptide chains because of accumulation of impurities (that is, amino acids) in each hydrolysis step.



N-terminal amino acid identified from phenylthiohydantoin

New N-terminal of shortened peptide chain

C-terminal Analysis

The C-terminal analysis is done even more efficiently by enzymatic methods rather than chemical methods. The *enzyme carboxypeptidase,* cleaves selectively the peptide linkage adjacent to C-terminal (free carboxylic group) in polypeptide chain. The removal of C-terminal residue gives rise to the formation of shortened polypeptide chain, which can be reacted with the *carboxypeptidase* enzyme to determine the new C-terminals.

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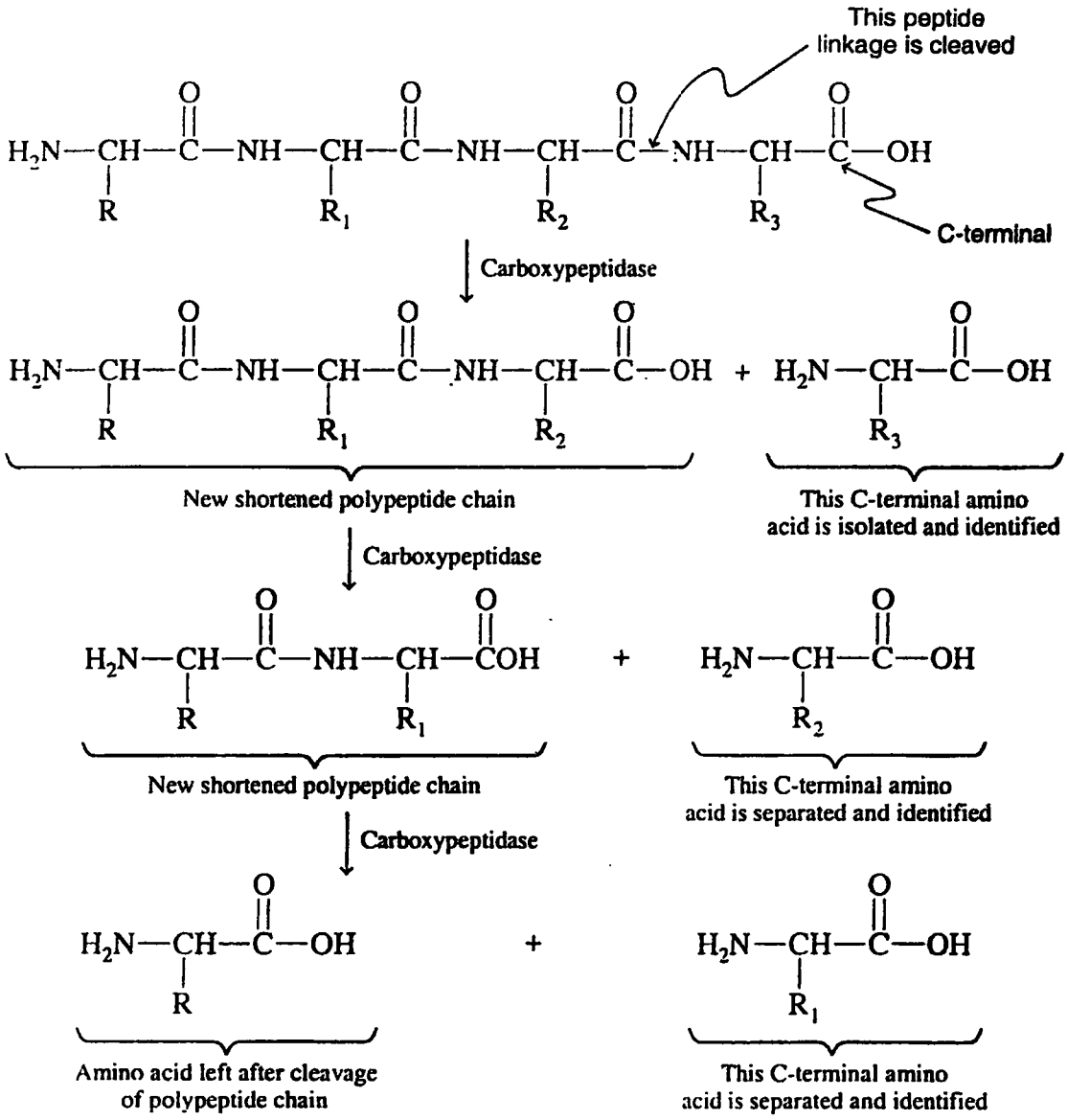

Sequence Analysis

The end group of terminal analysis can be employed for selective removal of terminal groups. However, it is a difficult process to identify a polypeptide chain by stepwise continuous removal of the terminal residues. The sequence of amino acids is identified by carrying out partial hydrolysis of the polypeptide chain. Partial hydrolysis breaks the chain selectively into smaller fragments that can be identified further. Specific enzymes are used which cleave the polypeptide at specific sites only. The smaller fragments can then be identified by Edman degradation.

Reagents/Enzymes	*Specific site of cleavage in polypeptide chain*
Cynogen bromide	Cleaves at carboxy end of methionine (Met)
Clostripain	Cleaves at carboxy end of arginine (Arg)

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Trypsin	Cleaves at carboxy end of arginine (Arg) and lysine (Lys)
Chymotrypsin	Cleaves at carboxy end of phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp)
Pepsin	Cleaves at carboxy end of aspartic acid (Asp), glutamic acid (Glu), leucine (Leu), phenyalanine (Phe), tyrosine (Tyr), and tryptophan (Trp)

Peptide Sequencing by Mass Spectrometry

Polypeptides of up to approximately 25 residues can be sequenced by the technique of *mass spectrometry* (MS), which involves an ionization technique called fast *atom bombardment* (FAB) in concert with a *tandem mass spectrometer* (two mass spectrometers coupled in series). The sequence of the polypeptide can be obtained from the molecular masses of the various fragments produced in the ionization stage in only a few minutes compared to the hour required for just one cycle of Edman degradation. In addition, mass spectrometry can be used to sequence several polypeptides in a mixture, alleviating the need to completely purify the sample prior to analysis. Other advantages of mass spectrometry are that it can be used to determine the sequence of peptides which have *blocked N-termini*, such as pyroglutamate, a derivative of glutamate in which the side chain carboxyl group forms an amide bond with its primary amino group (a common eukaryotic post-translational modification that prevents Edman degradation) and to characterize other *post-translational modifications* such as glycosylation and phosphorylation.

Recombinant DNA Technology

Although numberous proteins have been sequenced by Edman degradation using the above strategy, the determination of the sequences of large proteins by this method is a demanding and time-consuming process. Nowadays, *recombinant DNA technology* has enabled the sequences of even very large proteins to be determined by first sequencing the stretch of *DNA* encoding the protein and then using the *genetic code* to decipher the protein sequence. Even so, some direct protein sequence data is often required to confirm that the protein sequence obtained is the correct one. Thus, currently, protein sequencing and DNA sequencing are techniques that are used together to determine the complete sequence of a protein.

Information Derived from Protein Sequences

The amino acid sequence can provide information over and above the *primary structure* of the protein.

1. The sequence of interest can be compared with other known sequences to see whether there are similarities. For example, the sequences of hemoglobin and myoglobin indicate that they belong to the globin group or *family of proteins.*
2. The comparison of the sequences of the same protein in different species can provide information about *evolutionary relationships.*
3. The presence of repeating stretches of sequence would indicate that the protein may have arisen by *gene duplication* (*e.g.*, in antibody molecules).
4. Within the amino acid sequence there may be specific sequences which act as signals for the *post-translational processing* or the protein (*e.g.*, glycosylation or proteolytic processing).
5. The amino acid sequence data can be used to prepare *antibodies* specific for the protein of interest which can be used to study its structure and function.
6. The amino acid sequence can be used for designing *DNA probes* that are specific for the gene encoding the protein.

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PROTEINS

8.1 Introduction to Proteins

The term "protein", derived from the greek word *proteios* meaning *first,* was first of all used by Mulder in 1839 at the suggestion of Berzelius. However, *proteins are now defined as complex nitrogeneous substances which are found in the protoplasm of all animal and plant cells. Actually, the proteins are biopolymers containing large number of amino acids joined to each other by peptide bonds.*

Proteins are macromolecules which are constructred by the repetition of one or more structural elements, called the *monomers.* In the case of proteins, the monomers are a group of about 20 amino acids. These are linked together to form chains of varying length.

All proteins contain C, H, O and N, the presence of N distinguishing them from carbohydrates and fats. On an average proteins contain 16% nitrogen. Some proteins also have S in addition, and in a few proteins P and other elements may be present. The molecular weight of proteins varies from about 12,000 daltons (bovine insulin) to several million. Cells contain a very large number of proteins. The number may vary from 1000-2000 in the simplest bacteria to as many as 100,000 different proteins in human cells.

8.2 Classification of Proteins

There are several arbitrary classifications of the proteins in use. However, there is no satisfactory system of classification which depicts their differences and similarities. One can do the classification of proteins according to their structure, solubility behaviour or according to their non-protein moiety. Some of these systems of classification are as follows :

I. According to Solubility

According to this method, proteins may be divided into two groups :

1. Fibrous proteins : These are insoluble in common solvents but are soluble in concentrated acids and alkalis. These are highly resistant to digestion by proteolytic enzymes. These proteins appear as fibres made of linear molecules that are arranged roughly parallel to the fibre axis. The long linear protein chains are held together by intermolecular hydrogen bonds.

Fibrous proteins include the proteins of silk, wool, skin, hair, horn, nails, hoofs, quills, connective tissue and bone.

2. Globular proteins : These are soluble in water and in dilute acids, alkalis and salts. These proteins are more highly branches and cross-linked condensation products of basic or acidic amino acids. The polypeptide chains in this type of proteins are held together by cross linked groups or in an aggregate state. Such aggregates may also be folded to three dimensional structures by weak-non-covalent bonds.

Globular proteins include all the enzymes, oxygen carrying proteins, protein hormones, as well as other.

II. On the Basis of Increasing Complexity into their Structures

According to this system of classification, proteins may be divided into three main groups : simple, conjugated and derived proteins. We shall describe these one by one.

1. Simple proteins : These are such protein which upon hydrolsysis yield amino acids or their derivatives. These include the following groups :

(a) **Albumins :** These are soluble in water, coagulated by heat and precipitated by saturated salt solutions like ammonium sulphate. These are usually low or deficient in glycine. Examples of albumins are lactalbumin, serum albumin and egg albumin.

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(b) **Globulins :** These are soluble in dilute salt solutions of the strong acids and bases. These are insoluble in pure water or in moderately concentrated salt solutions. These are coagulated by heat. These are precipitated by half saturated their solutions with ammonium sulphate.

Globulins generally contain glycine. Examples of globulins are serum globulin, tissue globulin and vegetable globulin.

(c) **Glutelins :** These are soluble in dilute acids and alkalis. These are insoluble in neutral salts. These are coagulated by heat.

Glutelins are comparatively rich in arginine, proline and glutamic acid. Examples of glutelins are glutenin from wheat and oyrzenin from rice.

(d) **Prolamins :** These are soluble in 70-90 per cent ethanol. These are insoluble in absolute alcohol, water and other neutral solvents.

Prolamins contain large amounts of proline but are defficient in lysine. Examples of prolamins are zein from maize, gliadin from wheat and hordein from barley.

(e) **Albuminoids (Scleroproteins) :** These are insoluble in all neutral solvents and in dilute acids and alkalis. These are the proteins of supportive tissues. Examples of albuminoids are keratin from hair, hoof, etc. and fibroin from silk. These are attacked by enzymes.

Albuminoids are further subdivided into two types :

(i) Collagens : More than half the total protein in the mammalian body is collagen. When collagens are boiled with water, gelatin, a water soluble protein, is obtained. The collagens appear to be unique in their high content of hydroxyproline and in containing hydroxylysine. They are poor in sulphur, since cysteine and cystine are absent and contain no tryptophan. Collagens are found in skin, tendons and bones. These are attacked by pepsin or trypsin.

(ii) Elastins : These are present in tendon, arteries and other elastic tissues. Although similar to collagens in many respects, they cannot be converted into gelatins and are attacked slowly by trypsin.

(f) **Basic proteins :** These are strongly basic and are further divided into two subclasses.

(i) Histones : These are basic proteins which are soluble in water or dilute acids but are insoluble in dilute ammonia. These are not coagulated by heat. Histones are rich in basic amino acids like histidine and arginine but deficient in tryptophan and contain little cystine or methionine. Histones are readily hydrolysed by pepsin and trypsin.

Histones can be extracted in large amounts from certain glandular tissues, such as thymus and pancreas. Most histones are combined with nuclei acids, hemoglobins, etc.

(ii) Protamines : These are more basic than histones. These have a simpler structure of relatively low molecular weight. These are soluble in water or in NH_4OH. These are not coagulated by heat. They contain no sulphur but have a high nitrogen content (25-30 per cent) due to the presence of large quantities of arginine. Tyrosine and tryptophan are absent. Protamines are hydrolysed by enzymes like trypsin, papain but not by pepsin.

Typical protamines are salmine from salmon sperm, clupeine from herring and sturine from sturgeon. Protamines are principally found in egg cells.

2. Conjugated proteins : These are proteins which contain some non-protein substance called *prosthetic group*. This group may be separated from the protein part by carrying out hydrolysis very carefully. Sub-members of conjugated proteins are :

(a) **Nucleoproteins :** In these proteins, the prosthetic group is a nucleic acid. Examples of nucleoproteins are nuclein, nucleo histone from nuclei-rich material (*e.g.*, gladular tissue).

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(b) **Chromoproteins :** In these proteins, the prosthetic group is a chromophoric group called coloured prosthetic group. Examples of chromoproteins are hemoglobin, hemocyanin, cytochrome, flavoproteins.

(c) **Glycoproteins :** These are the proteins having carbohydrate phosthetic groups and these on hydrolysis yield amino sugars (hexoseamines). These are also known as mucoproteins.

Examples of glucoproteins are egg albumin, some serum albumins and certain serum globulins.

(d) **Phosphoproteins :** In these proteins, the prosthetic group possesses phosphoric acid in some form other than in the nucleic acids or in the lipoproteins. An example of phosphoproteins is caesin.

(e) **Lipoproteins :** These are water soluble proteins in which the prosthetic groups are such as phospholipid and cholesterol. Examples are several lipoproteins of serum.

(f) **Metalloproteins :** These are conjugated proteins that contain a metal which is an integral part of the structure. Metals found in metalloproteins are generally iron, magnesium, copper and manganese. Examples of metalloproteins are hemoglobin and chlorophyll. This is to be remembered that these are also included in chromoproteins.

3. Derived proteins : When proteins are hydrolysed by acids, alkalis or enzymes, the degradation products obtained from them are called derived proteins. The derived proteins are further classified on the basis of progressive cleavage as proteins, primary proteoses, secondary proteoses, peptones, polypeptides, simple peptides and amino acids.

Proteins ⟶ Denatured proteins
↓
Primary proteoses ⟶ Secondary proteoses.
(metaproteins)

Peptones ⟶ Polypeptides ⟶ Simple peptides ⟶ Amino acids.

Denatured proteins are insoluble proteins which are obtained by the action of heat on proteins.

Primary proteoses (also called metaproteins) are insoluble in water or dilute salt solution. However, they are soluble in acids or alkalis. These get precipitated by half-saturation with ammonium sulphate.

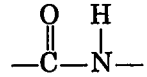
Secondary proteoses are soluble in water. They are precipitated by saturation with ammonium sulphate and are not coagulated by heat.

Peptones, polypeptides and simple peptides are soluble in water. They are all coagulated by heat. However, they are not precipitated by saturation with ammonium sulphate.

8.3 Chemical Structure of Proteins

Proteins are built up from smaller several units called *polypeptides*. These polypeptides are further made up of amino acids. The number of amino acids in a polypeptide and thus in a protein may vary considerably.

A peptide is composed of two or more amino acids joined through peptide bonds. The term peptide bond is applied to the amide link between amino acid residues.



When two amino acids combine to form a dipeptide, a water molecule is removed from the α-carboxyl group of one amino acid and the α-amino group of the other.

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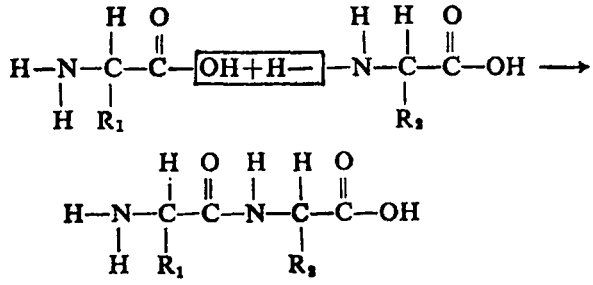

Similarly, tripeptides are formed by the linkage of three amino acids, tetrapeptides by the linkage of four amino acids and so forth. When many amino acids are joined together, the compound is called a *polypeptide*. The number of peptide bonds in a peptide is one less than the number of amino acid residues. By convention, a peptide chain is drawn with the amino acid residue containing the free ammonium group (the N terminal amino acid residue) on the left hand side and the residue containing the free carboxylate group (the C terminal amino acid residue) on the right hand side.

Infrared studies have been extensively used to characterise peptide linkage in peptides. For example, peptides generally show bands near 3300, 3100 cm^{-1}, and 1650, 1550 cm^{-1}. The bands near 3300 and 3100 cm^{-1} are characteristic of the hydrogen bonded N-H group whereas bands near 1650 and 1550 cm^{-1} are characteristic of the > C = O group.

There are many naturally occurring peptides which owe high biological significance. These include mammalian peptide hormones accessory growth factors and bacterial products. A summary of some biologically important peptides has been given in Table 8.1.

Table 8.1

POLYPEPTIDES OF BIOLOGICAL SIGNIFICANCE

	Peptide	*Biological Significance*	*Number of amino acid residues*
1.	Adreno-corticotropic hormone (ATCN)	Stimulates cortex of adrenal gland	39
2.	Bacitracin	Antibiotic	12
3.	Glucagon	Increases sugar in blood	29
4.	Glutathione	Biological reducing agent	3
5.	Gramicidin A	Antibiotic	10
6.	Melanocyte stimulating hormone (MSH)	Stimulates pigment cell production	
7.	Vasopressin	Water balance hormone	9

Some hormones are cyclic peptides rather than linear one. For example, oxytocin is a hormone in which amino acids are joined to form a circle which is held in its normal shape by involving covalent disulphide (S—S) bonds between cysteine molecules. Disulphide bonds are also present

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C-terminal end on the right. Then, the names of amino acids in a polypeptide are written from left to right, replacing the prefix—ine with—yl of that amino acid whose carboxy group has undergone reaction. If ambiguity is possible, peptide bonds are indicated by an arrow joining the abbreviations for the amino acids. The arrow points from the carbonyl to the amino group.

Although more than 100 amino acids are known only 20 of them participate in the synthesis of proteins. Plants have been found to synthesise all these amino acids from breakdown products of carbohydrates and reduced nitrogen. On the other hand, most animal cells could synthesise some of these amino acids.

In 1951 Sanger and his co-workers established the amino acid sequence of the hormone *insulin*. This was the first biologically active protein whose amino acid sequence has been determined. Insulin was found to consist of 51 amino acid residues. It is derived from an inactive precursor, *proinsulin*, with 84 amino acid residues. Removal of a piece containing 33 amino acid residues from the middle of the proinsulin polypeptide chain results in the formation of the two chains of the insulin molecule (Fig. 8.5). One of these chains, consisting of 21 amino acid residues, is called the α-*chain*, and the other chain consisting of 30 amino acid residues is called the β-*chain*. Stein and his co-workers later established the amino acid sequence of the enzyme *ribonuclease*.

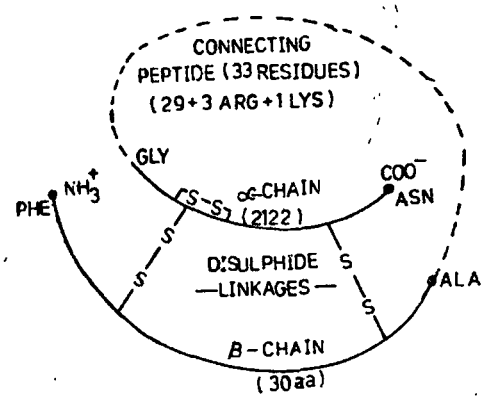

Fig. 8.5. Structure of insulin.

Chymotrypsinogen is composed of 246 amino acids (Fig. 8.6). The linear chain is held in a definite shape by 5 disulphide (S—S) bonds.

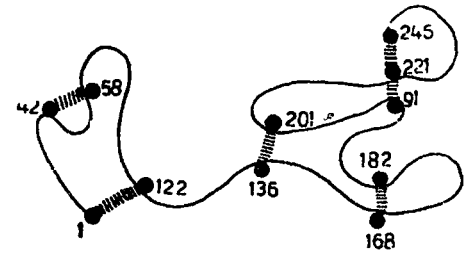

Fig. 8.6. Disulphide bonds in chymotrypsinogen.

Distinction between Polypeptides and Proteins

There is sharp dividing line between peptides and proteins. One arbitrary convention designates proteins as those molecules which are generally considered to have minimal chain length of about 100 amino acids corresponding to molecular weights above ~ 10,000 whereas peptides have less than 100 amino acids and a molecular weight below ~ 10,000. Some representative molecular weights of proteins are ribonuclease 13,7000, trypsin 23,800 hemoglobin 68,000 fibrinogen 450,000 thyroglobulin 630,000 and viruses, a few to several million.

Many people have accepted that a polypeptide chain contains usually units of only one amino acid whereas proteins of the body usually contain a combination of many, if not all, of the basic set of 20 amino acids and often modified derivatives of these as well.

8.4 Structural Configuration of Proteins or Physical Configuration of Proteins

Amino acids are arranged in various ways in a polypeptide chain. Further, the linear chains of polypeptides are arranged in space in various ways, thereby yielding different configurations of proteins. These different configurations give rise to the primary, secondary, tertiary and quaternary structures of proteins. These structures were initially worked out by two American scientists L. Pauling and Corey by using X-ray crystallography.

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1. Primary structure of proteins : The primary structure of a protein is mainly referred to the number, nature and sequence of amino acids along the peptide chains. The questions about primary structure which can be asked and which must be answered to provide an understanding of protein structure are as follows :

(*a*) What is the amino acid at the N-terminal end of the polypeptide chain (*i.e.*, NH_2 terminal) ?

(*b*) What is amino acid at the C-terminal (*i.e.*, COOH-terminal) end of the chain ?

(*c*) What amino acids occur between the amino acids in (*a*) and (*b*) ? How are they sequentially arranged ?

The answer to all the above questions can be obtained if the primary structure of protein is studied in a systematic manner. The various steps for establishing the primary structure for proteins are as follows :

(*a*) *Isolation of proteins :* The very first step is the isolation of protein from the tissues.

(*b*) *Separation and purification of proteins :* After isolation of proteins, the next step involves their separation by the following methods :

(*i*) Electrophoresis

(*ii*) Chromatography

(*iii*) Dialysis

(*iv*) Crystallisation.

(*c*) *Criteria of purity :* This step is essential to investigate whether the protein separated by the above methods is sufficiently pure or not. Several criteria are in use to test the purity of proteins but no single criterion ensures 100 per cent purity. Some of these criteria are as follows :

(*i*) Crystallinity

(*ii*) Electrophoresis

(*iii*) Ultracentrifuge method

(*iv*) Solubility method

(*v*) Biological activity

(*d*) *Number of peptide chains :* The next step is to ascertain whether the protein consists of a single peptide chain or whether it is composed of a number of subunits. If the latter, then the subunits are separated and each chain is examined separately.

(*e*) *Determination of amino acid composition :* The next step is to bring about the complete hydrolysis of the protein into its constituent amino acids and then their nature and amounts are determined.

From the relative amount of each of the amino acid present in a molecule, the empirical formula in terms of the amino acids of proteins can be deduced. The minimum molecular weight of the protein is determined from the empirical formula. The molecular weight of the protein is also determined by a physical method.

(*f*) *Determination of C-terminal amino acid :* Various methods are available to determine C-terminal amino acid of a protein.

(*g*) *Determination of N-terminal amino acid :* At the present time, a study of the N-terminal amino acid residues is almost a traditional beginning for the determination of sequence

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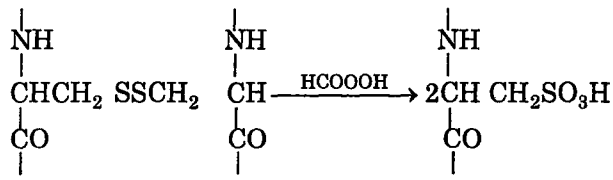
of amino acids in a peptide. If the N-terminal residues have been identified, it serves as a starting point for laying out the entire final sequence. Various methods are generally used for the determination of N-terminal amino acid in peptides (or proteins).

(*g*) *Determination of disulphide bond :* If a protein molecule contains the disulphide bond, it must be broken before amino acid sequence analysis is started. The disulphide bond can be broken either by oxidation or reduction. The oxidation is generally carried out with *performic acid* when there occurs the cleavage of disulphide bonds present in proteins (or peptides) to produce chains containing cysteic acid residues which are stable and assist the separation of the oxidation mixture by ion exchange methods.



(*h*) *Determination of the sequence of amino acids :* After establishing the identity of constituting amino acids and their relative amounts, the next problem is to ascertain the sequence of amino acids in a peptide. This is quite a difficult task because there are many possibilities in which constituent amino acids may be linked in the peptides. For example, a dipeptide composed of two different amino acids, A and B, may be combined in two different ways :

H_2N—A—CONH—B—COOH (I) H_2N—B—CONH—A—COOH (II)

On inspecting structures (I) and (II), it is found that the two ends of each molecule are different. The *amino-end* is called *N-terminal* and the *carboxy end* is called *C-terminal.*

Suppose we consider a tripeptide composed of amino acids A, B and C. These can be arranged in 3 or 3 × 2 × 1 or 6 different ways :

A.B.C. A.C.B. B.A.C. B.C.A. C.A.B. C.B.A.

If N terminal amino acid (N—T—AA) is determined by some method, the six possibilities are grouped into three pairs, *i.e.,* it is possible to know whether the tripeptide was either one or other or a pair. Similarly, the same possibilities arise if the C terminal amino acid (C—T—AA) is determined by some or other method.

Thus,

N-Terminal	*C-Terminal*
(*a*) A.B.C. and A.C.B.	(*d*) B.C.A. and C.B.A.
(*b*) B.A.C and B.C.A.	(*e*) A.C.B. and C.A.B.
(*c*) C.A.B and C.B.A.	(*f*) A.B.C. and B.A.C.

By determining both N– and C– terminal groups it is possible to determine the amino acid sequence of the tripeptide. For example if the N—T—AA determination reveals that the tripeptide belongs to group (*b*) and the C—T—AA determination reveals that the tripeptide belongs to group (*d*), the tripeptide is therefore B.C.A.

Suppose the application of N— and C—T—AA methods to tripeptide say, B—C—A, results in the removal of terminal amino acid. In such a situation, B.C.A. would be left with the following fragements.

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(*i*) N—T—AA first : Fragment C.A.

(*ii*) C—T—AA first : Fragment B.C.

If either of the above determinations are repeated, then the amino acid sequence in the tripeptide can be ascertained. It is to be remembered that the sequence of amino acids in a tripeptide may be determined by employing one method twice or by use of each method once.

Similarly, the sequence of the amino acids in the tetrapeptide may be determined by employing either the N—T—AA or C—T—AA method three times or by a combination of these methods (also three operations).

If the amino acids occur more than once in a protein or polypeptide chain, this will increase the difficulty in elucidating the amino acid sequence. In such cases, overlapping procedure is not of much help. However, different hydrolytic reagents are of much use in deciding the amino acid sequence in such cases because these can selectively break peptide bonds.

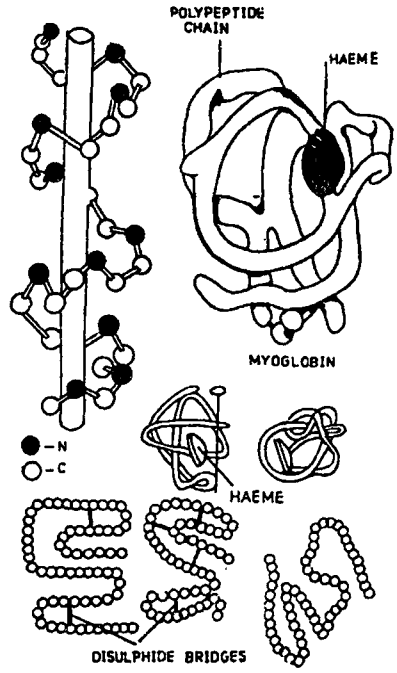

Fig. 8.7. Protein structure. *Top left*—A polypeptide chain in a helical configuration. *Top right*—The tertiary and quaternary structure of myoglobin. *Bottom*—Protein dematuration.

2. Secondary structure (II°) : Most long polypeptide chains are *folded* or *coiled* in a number of ways. This brings about a second level of organisation called the secondary structure. The most common form of coiling is the right handed *alpha helix*. Only right handed α-helix exists in nature since it is far more stable for L-amino acids than the left handed helix. In this helical form there are 3.6 amino acid residues per complete turn. The rise along the central axis is 1.5 Å per residue or 5.4°A for the pitch (repeat unit) (Fig. 8.8) The structure is stabilized by *intramolecular hydrogen bonds* between an amide *nitrogen* (—NH group) and the carboxyl *oxygen* (—C=O group) of the fourth amino acid residue away in the peptide chain.

$$C = O \text{———} H—N$$

Although the hydrogen bond is fairly weak, the large number of bonds involved maintains a stable structure. All the amino acid side chains are accommodated into the α-helix, since their side chains project away from the coil. The amino acids *proline* and *hydroxyproline* however, do not fit into the normal α-helix. They disrupt the α-helical structure and cause sharp bends or changes in the direction of the chain. In fact *proline* and *hydroxyproline*, along with *glycine, serine* and *asparagine* are called *'helix breaker's* because of this characteristic. The *α-keratins* making up the proteins of fur, claws, hooves and feathers consist mostly of α-helical chains. The α-helix is found in both fibrous and globular proteins.

Another form of secondary structure is the *β-conformation* β *pleated sheet*. This results from hydrogen bonding between two peptide chains. The chains may be *parallel* or *antiparallel*. In a *parallel chain* β *pleated sheet* the N atoms point in the same direction [Fig. 8.9(*a*)] while in the *antiparallel chain β-pleated sheet, alternate* chains are oriented in the same direction [Fig. 8.9 (*b*)]. The antiparallel structure permits maximum hydrogen bonding. The parallel chain structure

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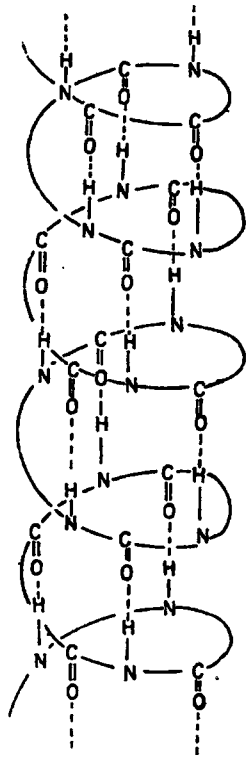

Fig. 8.8. Polypeptide chain in a helical configuration.

has less favourable hydrogen bonding arrangement. The β conformation is found in fibrous proteins. Most heat treatment and stretching of α-*keratin* converts it into β-*keratin* by breaking the stabilizing hydrogen bonds. An extended parallel chain β pleated sheet structure is formed. The antiparallel structure is found in *silk fibroin* which has a repeating sequence of 6 residues.

(Gly—Der—Gly—Ala—Gly—Ala)n.

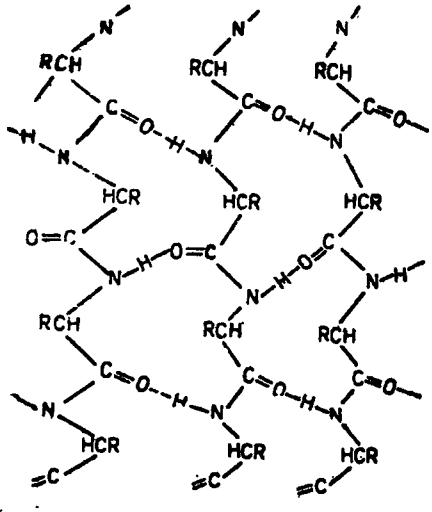

Fig. 8.9. (*a*) Parallel.

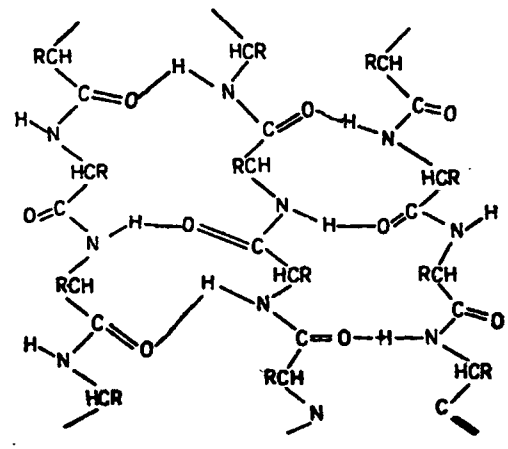

Fig. 8.9. (*b*) Antiparallel.

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3. Tertiary Structure (III°) : The polypeptide chain may undergo coiling and folding to produce the tertiary structure. The way the structure fold has an important bearing on the properties of the protein. The folding brings together *active amino acids,* which are otherwise scattered along the chain, and may form a distinctive *cavity* or *cleft* in which the substrate is bound. In proteins consisting of a single polypeptide chain, *e.g., myoglobin,* the tertiary structure determines the overall shape of the molecule. Thus proteins are called *globular proteins* if very compact and *fibrous proteins* form long thin threads.

The tertiary structure is maintained by a number of bonds which are of different types (Fig. 8.10.)

(*i*) *Disulphide bonds :* These are convalent links which give some rigidity to the protein molecule. They are relatively more stable than the other types of bonds. A disulphide bond is formed between two *cystein* residues. The enzyme *ribonuclease* consists of a single ploypeptide chain of 124 residues and contains four disulphide bonds. The disulphide bonds are oxidized by *performic acid,* which is therefore used to denature proteins or to separate polypeptide chains held together by S.S. bonds.

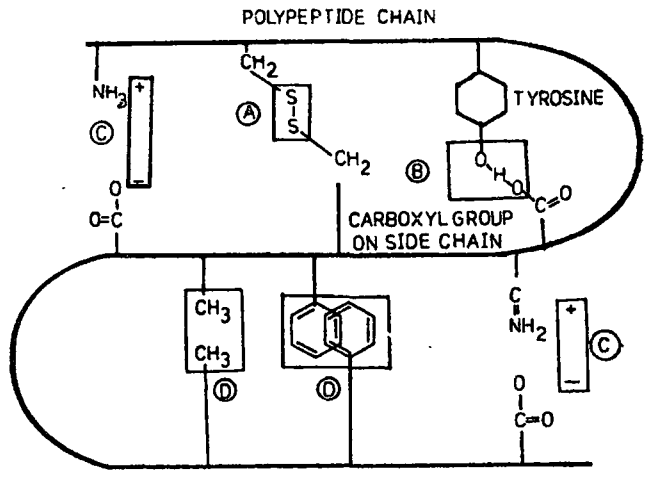

Fig. 8.10. Diagram of types of covalent bonds.
A. Disulphide bonds.
B. Hydrogen bonding.
C. Electrostatic interaction
D. Hydrophobic interaction of Monpolar side chains.

(*ii*) *Hydrogen bonds :* These are weak but since they are numerous they give considerable stability to the protein molecule. Hydrogen bonds can be formed by sharing *hydrogen* between *amide nitrogen* and *carbonyl oxygen* of the peptide backbone. They are also formed between groups present in the side chain. The side chains of aspartic and glutamic acids, serine and threonine can all form hydrogen bonds.

(*iii*) *Ionic or electrostatic bonds :* These are formed when an acidic and a basic amino acid are ionized and lie close together. Ionic bonds play an important role in binding a *basic protein* with an *acidic macromolecule, e.g.,* in the formation of nucleoproteins.

(*iv*) *Hydrophobic bonds :* These are formed because of the tendency of the non-polar side chains of neutral amino acids to closely associate with one another. When a protein molecule is submerged in an aqueous medium there is a tendency to expose a maximum number of its polar groups to the surrounding medium. Conversely a maximum number of non-polar groups are directed internally. Although hydrophobic interactions do not form true bonds they are of great importance in the formation of tertiary structure.

Structure of chymotrypsin : This has a tertiary structure which is shown in Fig. 8.11. This contains three polypeptide chains which are held together by disulphide bonds : The serine residue is at position 195, histidine 57 and aspartic acid 102. All the three groups occupy position close to each other in the three-dimensional structure (Fig. 8.11).

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4. Quaternary structure (IV°) is shown by proteins containing more than one polypeptide chain. Two or more polypeptide chains may associate to give rise to the quaternary structure. If the protein consists of identical units it is said to have a *homogeneous quaternary structure, e.g.,* the isozymes H_4 and M_4 of *lactic dehydrogenease* (LDH) and the enzyme *phosphorylase.* If the units are dissimilar the proteins is said to have a *heterogeneous quaternary structure, e.g., hemoglobin.*

Proteins such as hemoglobin, which consist of more than one polypeptide chain are said to possess quaternary structure. Proteins having this type of structure are said to be oligomeric and the individual polypeptide chains are known as protomers or subunits. Each of these subunits is characterised by its own secondary and tertiary structure. The subunits may or may not be identical. When subunits are held together by hydrogen bonds, they may be separated by reagents, (*e.g.,* water containing urea) which do not break covalent bonds.

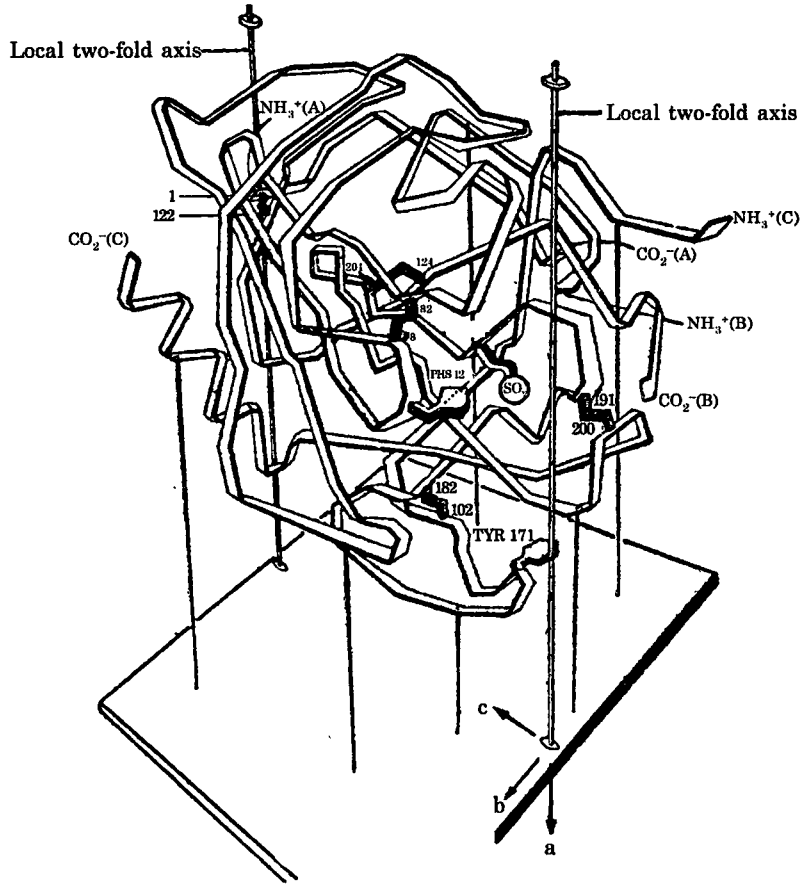

Fig. 8.11. Structure of chymotrypsin.

Hemoglobin contains four independent polypeptide chains, two identical α-chains and two identical β-chains. The chains in hemoglobin (also called *subunits*) are not held together by covalent bonds but by the interactions between the exposed groups of the folded chains. Each of these chains (or subunits) is characterised by its own secondary and tertiary structure.

Lactic dehydrogenase is another interesting example showing quaternary structure. This protein consists of four subunits having a molecular weight near 35,000. The subunits are

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somewhat asymmetrical themselves but form a rather cubelike aggregate by occupying positions at the corners of a square.

Formation of multienzyme complexes is closely related to the formation of quaternary structure for proteins. An interesting example of these is the pyruvate dehydrogenase of E coli. This is of molecular weight 4,000,000. This is composed of three types of enzymes, one of which, at least, is in turn composed of subunits. This can be spontaneously formed by mixing the component parts. The reassociation proceeds in high yield.

8.5 Hydrolysis of Proteins

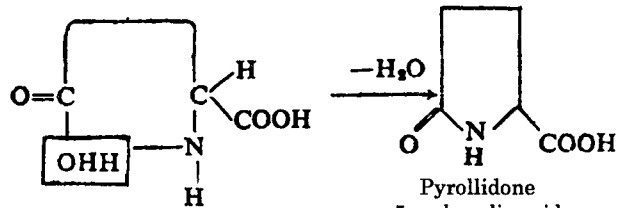
This can be carried out by the use of strong acid, strong base or proteolytic enzymes as catalysts. However, acid hydrolysis is the usual procedure.

(*a*) *Acidic hydrolysis :* This can be accomplished by a variety of strong acids. However, constant-boiling hydrochloric acid, about 6N, is most frequently employed. The acidic hydrolysis of proteins is generally carried out in evacuated sealed tubes at about 110°C for periods ranging from 12 to 96 hours. Under these conditions, the peptide bonds are quantitatively hydrolysed to yield the amino acid hydrochlorides. Undesirable side-effects of acidic hydrolysis are as follows :

(*i*) Among the amino acids that usually occur in proteins, all the tryptophan and variable amounts of cysteine and cystine are slowly destroyed. Serine and threonine are slowly destroyed. Formation of *humin*, a black polymer of breakdown product of tryptophan, acccompanies acid hydrolysis.

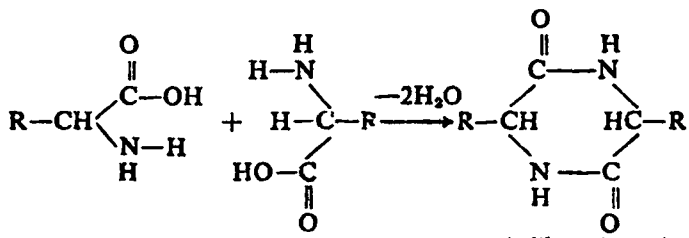
(*ii*) Glutamic and asparagine are deamidated to glutamate and aspartate.

(*iii*) Glutamic acid undergoes intramolecular dehydration to pyrrolidone 5-carboxylic acid.



Glutamic acid — Pyrollidone 5-carboxylic acid

(*iv*) Other amino acids may undergo intermolecular dehydration forming cyclic anhydrides or diketopiperazines.



Two amino acids — A diketopiperazine

In spite of these difficulties, acidic hydrolysis is ordinarily the method of choice because the amino acids are not racemised by acid hydrolysis.

(*b*) *Alkaline hydrolysis :* The complete hydrolysis of proteins may also be carried out by incubating them with 2 to 4N sodium hydroxide or 5N barium hydroxide at 100°C for 4 to 8 hours. Such hydrolysis is of limited use due to the following difficulties :

(*i*) Cysteine, cystine, serine, threonine and arginine are destroyed in this process.

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(*ii*) Other amino acids may be partially destroyed by deamination.

(*iii*) Racemisation of amino acids occurs.

In spite of the above mentioned difficulties, the alkaline hydrolysis is useful for the analysis of tryptophan because it is not destroyed during alkaline hydrolysis.

(*c*) *Enzymatic hydrolysis :* This technique is very valuable for the elucidation of amino acid sequence in a protein. However, the complete enzymatic hydrolysis is slow and difficult because most enzymes attack only specific types of linkages rapidly. Often, by use of a number of enzymes each in turn, it is possible to degrade the protein into smaller and smaller fragments and ultimately into the constituent amino acids.

The bacterial peptidases *subtilisin* and *pronase* catalyse hydrolysis of all peptide bonds but the reaction is slow compared to acid catalysed hydrolysis. Other proteolytic enzymes (*trypsin, chymotrypsin*), catalyse hydrolysis of certain peptide bonds quite rapidly. The enzymatic hydrolysis is complicated, owing to possible contamination resulting from proteolysis of the enzymes themselves.

8.6 Properties of Proteins

Some of the characteristic properties of proteins are as follows :

Denaturation and renaturation : All the proteins are optically active and precipitated from aqueous solution by heating, by standing in acid or alkaline solution, by treatment with organic solvents such as ethanol or acetone, by high concentration of urea, by detergents, and by physical means such as X-ray, light and shaking. Proteins in this precipitated state are said to be *denatured* and the process of reaching this state is called *denaturation* which occurs most readily near the isoelectric point.

Characteristic of denaturation : These are as follows :

(*i*) Denaturation of a protein involves changes in the molecule that leads to changes in physical properties such as decreased solubility, changes in optical rotation and generally the loss of solubility activity, *e.g.*, enzymes (all are proteins) become inactive when denatured. *Denaturation is now considered to be the result of changes in conformation of unfolding of the protein molecule, i.e.,* the secondary and tertiary structures of proteins are completely lost in denaturation without any break in the primary structure.

(*ii*) The chemical reactivity of the various functional groups in the denatured protein is more than that in the native protein. The reason for this is the opening up of various functional groups in the denatured protein.

(*iii*) Denaturation of proteins is usually irreversible. However, examples are known in which denaturation has been found to be reversible. This reversal of denaturation is generally called *renaturation* or *refolding*. For example, hemoglobin can be denaturated in acid solution and the process is reversed by neutralisation under the correct conditions.

Suppose the denaturation has been carried out by heating. In that case, no renaturation takes place on rapid cooling. However, if cooling is done very slowly under carefully controlled conditions, renaturation takes place. In these circumstances, the process of renaturation has been referred to as *annealing.*

An interesting example of irreversible or uncontrolled denaturation is the boiling of an egg during which there occurs the destroying of tertiary structure of protein in an irreversible manner to form a disorganised mass of polypeptide chains (Fig. 8.12).

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An interesting example of reversible or controlled denaturation is the treatment of a protein molecule with urea and mercaptoethanol during which there occurs the breakage of disulphide links and also the uncoiling of the polypeptide chains. The denaturated protein on slow reoxidation without the urea is again changed into the original tertiary structure (Fig. 8.13).

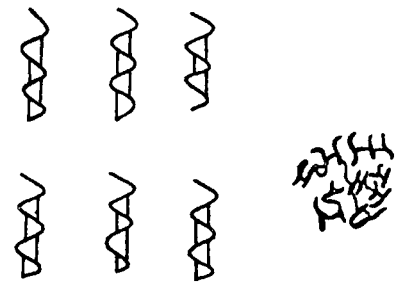

Fig. 8.12. Egg albumin before cooling. After cooking.

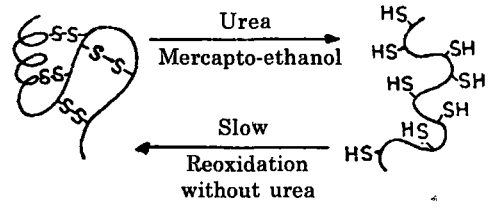

Fig. 8.13.

(*iv*) The isoelectric pH of denatured proteins is higher than that of the parent substance; they have less capacity to combine with water, they are more easily digestible and they are biologically inactive.

(*v*) *Isoelectric point :* The properties of proteins as electrolytes are determined by the ionisable groups in the molecule. As each protein chain contains one free α-amino acid and one free-end-carboxyl group for each protein chain, most of the ionisable groups must come from the R-groups. Due to the presence of these ionisable groups in the protein chains, the proteins also have some definite isoelectric pH at which they do not migrate in an electric field. At the isolectric pH, the number of positive charges is equal to the negative charges, giving a net charge of zero.

The isoelectric pH of the proteins has been found to depend upon the relative number of acidic or basic groups, which are rendered by amino acids. Serum albumin, which has many acidic amino acids has an isoelectric pH of 4.7. Gliadin with many basic amino acids has 9.0 isoelectric pH. Isoelectric pH of certain other proteins have been given in Table 8.2.

Table 8.2

ISOELECTRIC pH OF SOME PROTEINS

Protein	*pH*	*Protein*	*pH*
Casein	4.6	Cytochrome c	9.8
Avidin	10.0	Tetanus toxin	5.1
Lysozyme	11.0	Serum globulin	5.4
Pepsin	2.7	Edestin	6.9

At the isoelectric pH, the physical properties of a protein are at a minimum. For example, mobility in an electric field, osmotic pressure, swelling capacity, viscosity, and solubility are minimal at this isoelectric pH. These minimal properties are useful for determining the isoelectric pH.

Proteins are cations at pH values lower than the isoelectric pH and anions at pH values higher than the isoelectric pH.

Coagulation of proteins : Most of the proteins are hydrophilic colloids. However, few exceptions are insulin, tobacoo mosaic virus, etc. These are crystalline. Proteins are precipitated (or coagulated) in solution alkaline to the isoelectric pH by positive ions such as Zn^{2+}, Cd^{2+}, Hg^{2+}, Fe^{3+}, Ca^{2+}, and Pb^{2+}. At this pH the protein has a negative charge.

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Negative ions combine with proteins in solutions more acidic than the isoelectric pH to form salts. The common precipitants in this group are trichloroacetic acid, tungstic acid, phosphotungstic acid, sulphosalicylic acid, picric acid and tannic acid.

Salting in and out of proteins : The solubility of many proteins is increased in the presence of small concentrations of various neutral salts. This is referred to as *salting in* of proteins.

The phenomenon of *salting in* of proteins is probably caused by forces of attraction between salt and protein at low salt concentation, leading to increased solubility.

As the concentration of the neutral salt is increased, the solubility increases to maximum and then starts decreasing and finally the protein is precipitated. This is referred to as *salting out*.

The phenomenon of *salting out* of proteins is probably caused due to the competition for water between the protein and the salt at high concentrations.

The salting out of proteins is an effective method of purification.

Oxidation of proteins : Proteins get oxidized by putrefaction processes to form the products such as amino nitrogen compounds, CO_2 and water depending upon conditions employed. The bad smell of the dead and the decaying bodies is attributed to the putrefaction of the protein.

8.8 Protein Folding

Under appropriate physiological conditions, proteins *spontaneously fold* into their native conformation. As there is no need for external templates, this implies that the primary structure of the protein dictates its three-dimensional structure. From experiments with the protein *RNase_A* it has been observed that it is mainly the internal residues of a protein that direct its folding to the native conformation. Alternation of surface residues by mutation is less likely to affect the folding than changes to internal residues. It has also been observed that protein folding is driven primarily by *hydrophobic forces*. Proteins fold into their native conformation through an *ordered set of pathways* rather than by a random exploration of all the possible conformations until the correct one is stumbled upon.

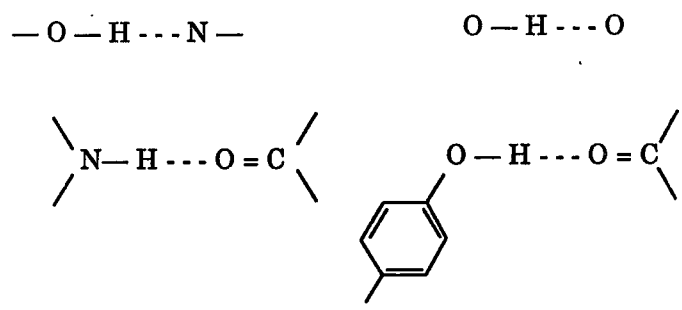

Fig. 8.14. Examples of hydrogen bonds (shown as dotted lines).

Although proteins can fold *in vitro* (in the laboratory) without the presence of accessory proteins, this process can take minutes to days. *In vivo* (in the cell) this process requires only a few minutes because the cells contain *accessory proteins* which assist the polypeptides to fold to their native conformation. There are three main classes of protein folding accessory proteins :

(*i*) *Protein disulfide isomerases* catalyze disulfide interchange reactions, thereby facilitating the shuffling of the disulfide bonds in a protein until they achieve their correct pairing.

(*ii*) *Peptidyl prolyl cis-trans isomerases* catalyze the otherwise slow inter-conversion of Xaa-Pro peptide bonds between their *cis* and *trans* conformations, thereby accelerating the folding of Pro-containing polypeptides. One of the classes of peptidyl prolyl *cis-trans* isomerases is inhibited by the *immuno-suppressive drug* cyclosporin A.

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(*iii*) *Molecular chaperones,* which include proteins such as the heat shock proteins 70 (Hsp 70), the chaperonins and the nucleoplasmins. These prevent the improper folding and aggregation of proteins that may otherwise occur as internal hydrophobic regions are exposed to one another.

8.9 Protein Structure Determination

The three-dimensional structure of a protein can be determined almost to the atomic level by the techniques of *X-ray crystallography* and *nuclear magnetic resonance (NMR) spectroscopy.* In X-ray crystallography a crystal of the protein to be visualized is exposed to a beam of X-rays and the resulting diffraction pattern caused as the X-rays encounter the protein crystal is recorded on photographic film. The intensities of the diffraction maxima (the darkness of the spots on the film) are then used to mathematically construct the three-dimensional image of the protein crystal. NMR spectroscopy can be used to determine the three-dimensional structures of small (up to approximately 30 kDa) proteins in aqueous solution.

8.10 Collagen

Introduction : Collagen is the name given to a family of structurally related proteins that form strong insoluble fibers. Collagens consist of three polypeptide chains, the identity and distribution of which vary between collagen types. The different types of collagen are found in different locations in the body.

The collagen polypeptides are post-translationally modified by hydroxylation and glycosylation on transport through the rough endoplasmic reticulum and Golgi. The three polypeptides form the triple-helical procollagen which is screted out of the cell. The extension peptides are removed to form tropocollagen which then aggregates into a microfibril and is covalently cross-linked to form the mature collagen fiber.

Composition and Post-Translational Modifications

One-third of the amino acid residues in collagen are Gly, while another quarter are Pro. The hydroxylated amino acids 4-hydroxyproline (Hyp) and 5-hydroxylysine (Hyl) are formed post-translationally by the action of proline hydroxylase and lysine hydroxylase. These Fe^{2+}-containing enzymes require ascorbic acid (vitamin C) for activity. In the vitamin C deficiency disease scurvy, collagen does not form correctly due to the inability to hydroxylate Pro and Lys. Hyl residues are often post-translationally modified with carbohydrate.

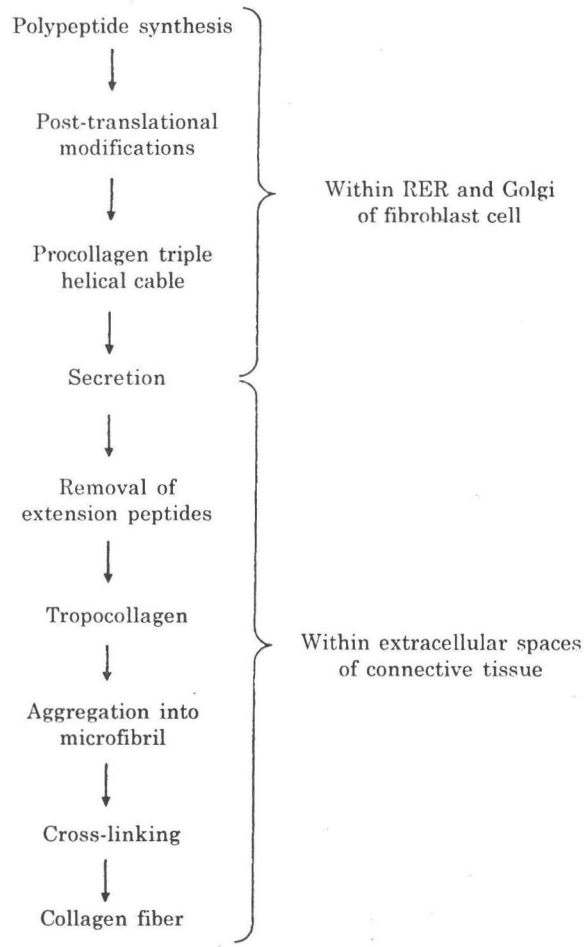

Fig. 8.15. Overview of the biosynthesis of collagen.

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Structure : Collagen contains a repeating tripeptide sequence of Gly-X-Y, where X is often Pro and Y is often Hyp. Each polypeptide in collagen folds into a helix with 3.3 residues per turn. Three polypeptide chains then come together to form a triple-helical cable that is held together by hydrogen bonds between the chains. Every third residue passes through the center of the triple helix, which is so crowded that only Gly is small enough to fit. One form of osteogenesis imperfecta (brittle bones) is caused by the mutation of Gly residue to another amino acid, which prevents the triple-helical cable folding correctly and results in defective collagen.

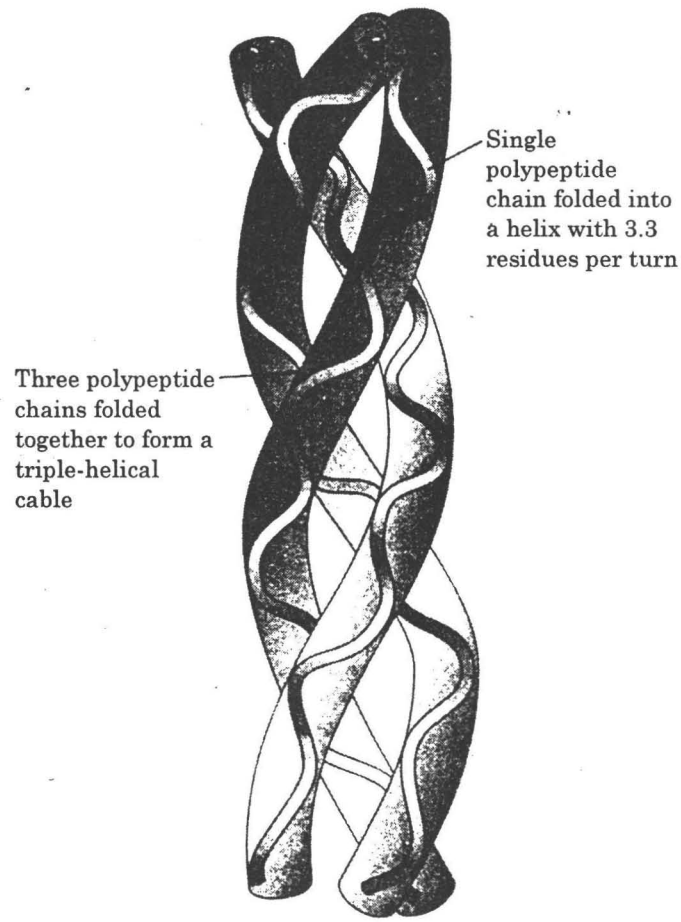

Fig. 8.16. Arrangement of the three polypeptide chains in collagen.

Secretion and Aggregation: The extension peptides on both the N and C termini of the polypeptide chains direct the formation of the triple-helical cable and prevent the premature aggregation of the procollagen molecules within the cell. Following secretion out of the cell, the extension peptides are cleaved off by peptidases, and the resulting tropocollagen molecules aggregate together in a staggered array.

Cross links : Covalent cross-links both between and within the tropocollagen molecules confer strength and rigidity on the collagen fiber. These cross-links are formed between Lys and its aldehyde derivative allysine. Allysine is derived from Lys by the action of the copper-containing lysyl oxidase which requires pyridoxal phosphate for activity. The disease lathyrism is caused by the inhibition of lysyl oxidase by the chemical β-aminopropionitrile in sweet pea seeds, and results in defective collagen due to the lack of cross-links.

Bone formation : The spaces between the ends of the tropocollagen molecules in a collagen fiber (see Fig. 8.17) are the *nucleation sites* for the deposition of a form of *calcium phosphate, hydroxyapatite,* in bone information. Further hydroxyapatite is added until the nucleation sites grow and join with one another to form the mature bone structure.

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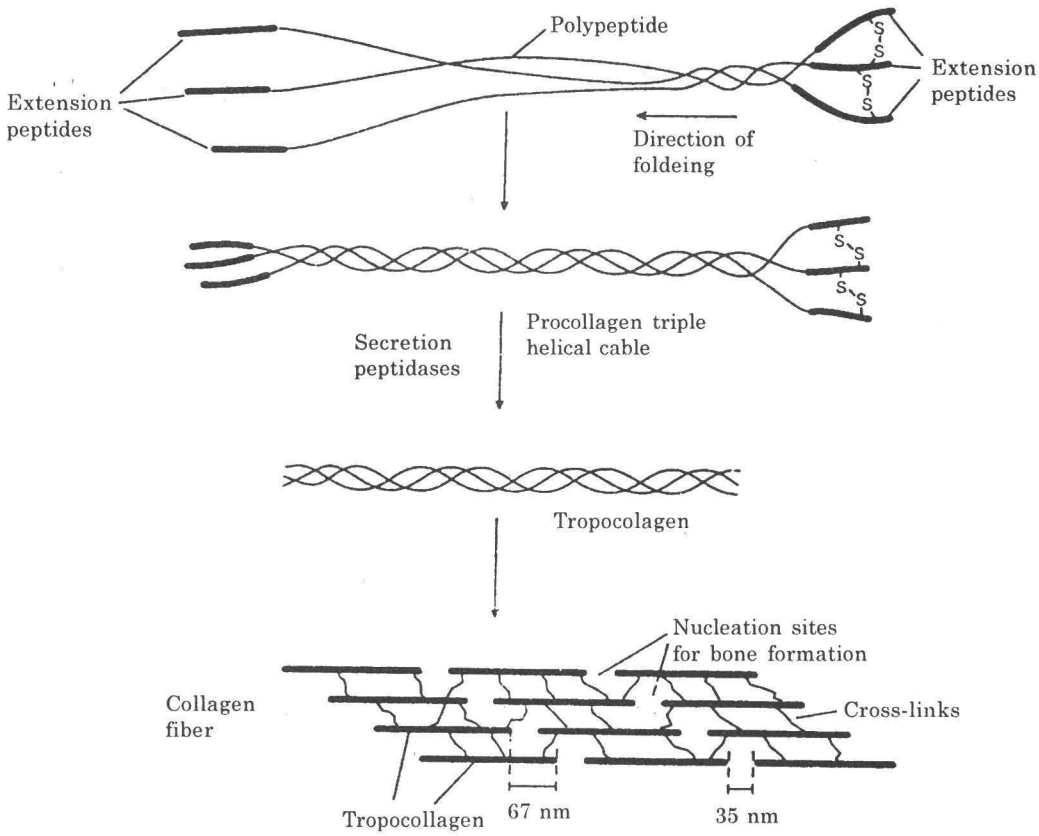

Fig. 8.17. Role of the extension peptides in the folding and secretion of procollagen. Once secreted out of the cell, the extension peptides are removed and the resulting tropocollagen molecules aggregate and are cross-linked to form a microfibril.

8.11 Myoglobin and Hemoglobin

Hemoglobin and myoglobin are the two oxygen-binding proteins present in large multicellular organisms. Hemoglobin transports oxygen in the blood and is located in the erythrocytes; myoglobin stores the oxygen in the muscles.

1. *Myoglobin :* It was the first protein to have its three-dimensional structure solved by X-ray crystallography. It is a globular protein made up of a single polypeptide chain of 153 amino acid residues that is folded into eight α-helices. The heme prosthetic group is located within a hydrophobic cleft of the folded polypeptide chain.
2. *Haemoglobin :* It has quaternary structure as it is made up of four polypeptide chains; two α-chains and two β-chains ($\alpha_2\beta_2$), each with a heme prosthetic group. Despite little similarity in their primary sequences, the individual polypeptides of hemoglobin have a three-dimensional structure almost identical to the polypeptide chain of myoglobin.

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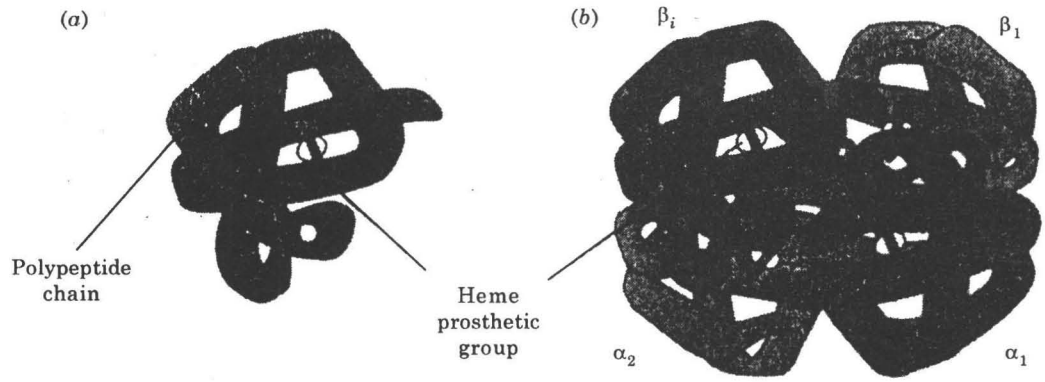

Fig. 8.18. Structure of (*a*) myoglobin and (*b*) hemoglobin, showing the α and β polypeptide chains.

Binding of oxygen to heme : The heme prosthetic group consists of a protoporphyrin IX ring and a central Fe^{2+} atom which forms four bonds with the porphyrin ring. In addition, on one side of the porphyrin ring the Fe^{2+} forms a bond with the proximal histidine (His F8); a residue eight amino acids along the F-helix of hemoglobin. The sixth bond from the Fe^{2+} is to a molecule of O_2. Close to where the O_2 binds is another histidine residue, the distal histidine (His E7), which prevents carbon monoxide binding most efficiently.

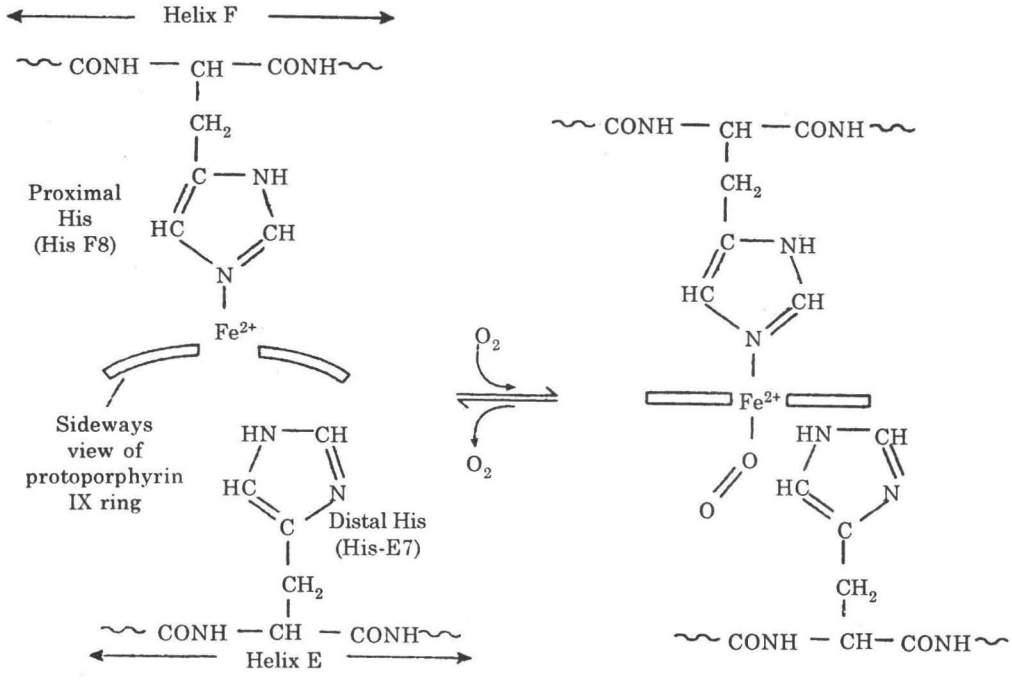

Fig. 8.19. Binding of O_2 to heme. The Fe^{2+} of the protoporphyrin ring is bonded to His F8 but not to His E7 which is located nearby. As the heme Fe^{2+} binds O_2, helix F moves closer to helix E.

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Allostery : Hemoglobin is an *allosteric protein.* This means that the binding of O_2 to one of the subunits is affected by its interactions with the other subunits. In fact the binding of O_2 to one hemoglobin subunit induces conformational changes (see Fig. 8.19) that are relayed to the other subunits, making them more able to also bind O_2 by raising their affinity for this molecule. Thus binding of O_2 to hemoglobin is said to be *cooperative.* In contrast, the binding of O_2 to the single polypeptide unit of myoglobin is *non-cooperative.* This is clearly apparent from the *oxygen dissociation curves* for the two proteins : that for hemoglobin is *sigmoidal,* reflecting this cooperative binding, whereas that for myoglobin is *hyperbolic* (Fig. 8.20). From the O_2 dissociation curve it can also be seen that for any particular oxygen pressure the degree of saturation of myoglobin is higher than that for hemoglobin. In other words, myoglobin has a higher affinity for O_2 than does hemoglobin. This means that in the blood capillaries in the muscle, for example, hemoglobin will release its O_2 to myoglobin for storage there.

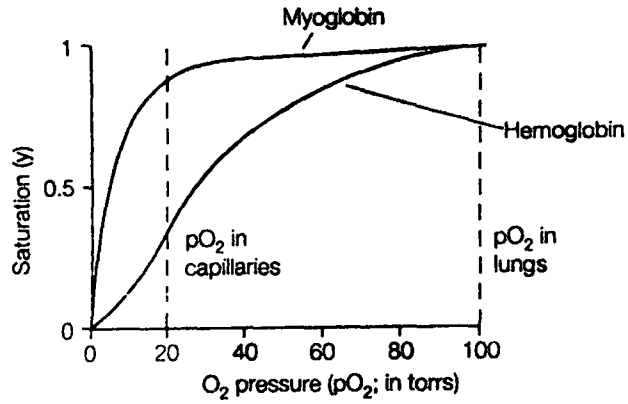

Fig. 8.20. Oxide dissociation curves for hemoglobin and myoglobin.

Mechanism of the allosteric change : X-ray crystallogrphy revealed that *oxyhemoglobin,* the form that has four O_2 molecules bound, differs markedly in its quaternary structure from deoxyhemoglobin, the form with no O_2 bound. In the absence of bound O_2, the Fe^{2+} lies slightly to one side of the porphyrin ring, which itself is slightly curved (Fig. 8.19). As a molecule of O_2 binds to the heme prosthetic group it pulls the Fe^{2+} into the plane of the porphyrin ring (Fig. 8.19), The binding of O_2 to hemoglobin is affected by the concentration of H^+ ions and CO_2 in the surrounding tissue; the Bohr effect. In actively metabolizing tissue, such as muscle, the concentrations of these two substances are relatively high. This effectively causes a shift of the O_2 dissociation curve for hemoglobin to the right, promoting the release of O_2. This comes about because there are H^+ binding sites, primarily His 146 in the β-chain, which have a higher affinity for binding H^+ in deoxyhemoglobin than in oxyhemoglobin. An increase in CO_2 also causes an increase in H^+ due to the action of the enzyme *carbonic anhydrase* which catalyzes the reaction :

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$$

In addition, CO_2 can react with the primary amino groups in the polypeptide chain to form a negatively charged carbamate. Again, this change from a positive to a negative charge favours the conformation of deoxyhemoglobin. On returning in the blood to the lungs, the concentrations of H^+ and CO_2 are relatively lower and that of O_2 higher, so that the process is reversed and O_2 binds to hemoglobin. Thus, it can be seen that not only does hemoglobin carry O_2 but it also carries CO_2 back to the lungs where it is expelled.

2, 3-Bisphosphoglycerate is a highly anionic organic phosphate molecule (Fig. 8.21) that is present in erythrocytes along with the hemoglobin. This molecule promotes the release of O_2 from

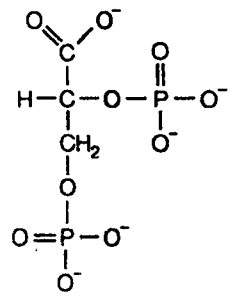

Fig.8.21. 2,3-Bisphosphoglycerate.

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hemoglobin by lowering the affinity of the protein for O_2. 2,3-Bisphosphoglycerate binds in the small cavity in the center of the four subunits. In oxyhemoglobin this cavity is too small for it, whereas in deoxyhemoglobin it is large enough to accommodate a single molecule of 2, 3-bisphosphoglycerate. On binding in the central cavity of deoxy-hemoglobin it forms ionic bonds with the positively charged amino acid side-chains in the β-subunits, stabilizing the quaternary structure. H^+, CO_2 and 2,3-bisphosphoglycerate are all *allosteric effectors* as they favour the conformation of deoxyhemoglobin and therefore promote the release of O_2. Because these three molecules act at different sites, their effects are additive.

Hemoglobinopathies

Comparison of hemoglobin sequences from different species reveals that only nine amino acid residues are invariant. Some residues are subject to conservative substitution of one residue by another with similar properties, others to nonconservative substitution where one amino acid residue is replaced by another with different properties. Hemoglobinopathies are diseases caused by abnormal hemoglobins. The best characterized of these is the genetically transmitted, hemolytic disease sickle-cell anemia. This is caused by the non-conservative substitution of a glutamate by a valine, resulting in the appearance of a hydrophobic sticky patch on the surface of the protein. This allows long aggregated fibers of hemoglobin molecules to form which distort the shape of the red blood cells. Heterozygotes carrying only one copy of the sickle-cell gene are more resistant to malaria than those homozygous for the normal gene.

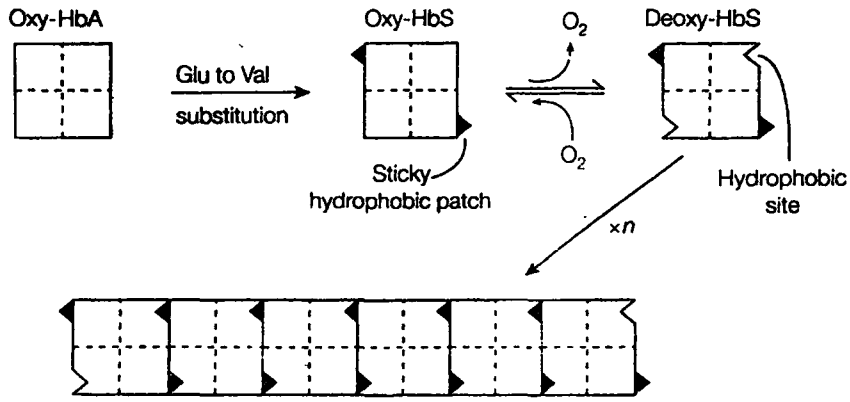

Fig. 8.22. Molecular basis for the aggregation of deoxyhemoglobin molecules in sickle-cell anemia.

8.12 Souring of Milk (Denaturation of Milk Proteins)

Sometimes milk when left without refrigeration for longer periods develops a sour taste and gets coagulated (curdled). This phenomenon is called 'souring of milk' and is an example of denaturation of proteins.

The bacteria present in the milk produce lactic acid as a metabolic waste product. An increase in the lactic acid content causes lowering of pH of milk (increased acidity). At a pH of 4.6, the major milk protein casein precipitates as it reaches its isoelectric point and forms curd.

Milk can also be curdled by adding lemon juice or vinegar (CH_3COOH) as these cause precipitation of casein due to lowering of pH. The curdled milk is further used to prepare cheese by heating.

Milk Protein—An Antidote for Heavy Metal Ions Poisoning (Hg^{2+}, Ag^{2+}, Pb^{2+})

Poisoning due to heavy metal ions occurs due to their ability to form strong covalent bonds

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with carboxylate group and sulfur atoms (in disulfide linkages) of proteins causing their denaturation. If these metal ions go into the system accidentally, their circulation in the system is prevented by giving milk as an antidote. The milk proteins readily combine with metal ions and form insoluble complex with metals and get coagulated (denaturation of protein) in the stomach. This is followed by administration of vomit inducing (emetic) drug as the action of gastric juices may destroy the protein and re-liberate metal ions. The emetic removes the metal ions containing coagulated milk proteins from stomach. Raw egg white is also used as an antidote for heavy metal poisoning.

8.13 Biological Importance of Proteins

Proteins constitute a large part of the structure of cells and are present in all tissues. Many proteins also have special physiological functions.

(1) Membrane proteins : Proteins and lipids form the major structural components of cell membranes. The membrane associated proteins consist of *integral* or *intrinsic* proteins and *peripheral* or *extrinsic* proteins. The *integral* proteins include *translocases* which regulate the movement of substances across the membrane. The *peripheral* proteins include *cytochrome c* and *monoamine oxidase.* Many enzymes and enzyme systems are associated with the membrane, *e.g.,* the components of the electron transport chain.

(2) Enzymes : These are special proteins produced with an organism which are capable of catalysing specific catalytic reactions. They are *biocatalysis* which influence the rate of a chemical reaction, usually without undergoing any change themselves. All enzymes are proteins. Over a thousand enzymes are known. They are of great biological importance since they are involved in the bisynthesis of macromolecules. *Flavoproteins* are conjugated protein (protein + Flavin) enzymes containing the vitamin riboflavin. *Metalloproteins* (metal ion or ions + Protein) are enzymes containing mineral elements.

Table. 8.3

PEPTIDE AND PROTEIN HORMONES

Pituitary	*Alimentary canal*
1. Somatotropin (growth hormone, GH)	13. Enterogastrone
2. Adrenocorticotropic hormone, (ACTH)	14. Cholecystokinin
3. Thyrotropin (thyroid stimulating hormone, TSH)	15. Secretin
4. Follicle stimulating	16. Pancreozymin
5. Luteinizing hormone (LH)	*Thyroid*
6. Prolactin	17. Calcitonin
7. Oxytocin	*Parathyroid*
8. Vasopressin	18. Parathormone (parathyrin)
9. Melantropins (MSH)	*Kidney*
Pancrease	19. Renin
10. Insulin	20. Erythropoietin
11. Glucagon	*Ovary*
12. Gastrin	21. Relaxin

(3) Hormones : Several hormones are peptides and proteins. They play an important role in the regulation of metabolic reaction. See Table 8.1.

(4) Blood proteins : The blood proteins include the *plasma proteins* and *haemoglobin.*

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(5) Plasma proteins : Electrophoresis at pH 8.6 in barbital buffer separates, *γ-globulin* and *fibrinogen*. Starch-gel electrophoresis breaks up the bands into 20 zones containing 50 plasma proteins.

(6) Albumin (MW 76,000) is synthesized in the liver and is the most abundant plasma protein. It maintains the colloid osmotic pressure of plasma and takes part in the transport of a variety of materials. It carries substances like bilirubin and some drugs which are otherwise insoluble in plasma.

(7) α_1*-Chymotrypsin* (MW 45,000) inhibits the proteolytic action of protein digesting enzymes like trypsin, chymotrypsin and plasmin.

(8) Hapatoglobin is an α2-globulin. When R.B.C. are damaged, hemoglobin released into the plasma is bound by haptoglobin and thus prevented from beings excreted through the urine.

(9) α_2*-Macoglobulin* (MW 820,000) is an inhibitor of proteolytic enzymes.

Transferrin (MW 90,000) is a β-globin involved in the transfer of Fe in plasma.

Gamma (γ) globulins *(immunoglobulins, antibodies)* are produced in the spleen and lymphatic cells in response to foreign substances (antigens). There are five classes of immunoglobulines, IgG, IgA, IgM, IgD, and IgE.

Fibrinogen, a precursor of fibrin, is formed during the clotting of blood.

Lipoproteins are of four types :

(*i*) High Density Lipoproteins (HDL)—(α-lipoproteins)

(*ii*) Low Density Lipoproteins (LDL)—(β-lipoproteins)

(*iii*) Very Low Density Lipoproteins (VLDL)—(pre β-lipoproteins)

(*iv*) Chylomicrons—the principal form in which lipids absorbed in the inestine are made soluble in the plasma.

Lipoproteins are concerned with transport of fat in blood.

Complement : The complement system consists of heat labile substances in the serum. They cause lysis of R.B.C. and destruction of certain bacteria in the presence of an appropriate antibody. There are 9 components, C'1 to C'9.

Hemoglobin is a conjugated protein consisting of globin + haeme (an Fe pophyrin prosthetic group). It consists of 4 polypeptide chains, 2 alpha chains and 2 beta chains. Each chain has a molecular weight of 16,750 and is attached to one atom of iron.

(5) Antibiotics : Some antibiotics like gramicidin S, tyrocidin and penicillin G are peptides.

(6) Nucleoproteins are conjugated proteins (Protein + Nucleic acid) of cell nuclei. The proteins include *protamines, histones* and *non-histone chromosomal (NHC) proteins.*

(7) Multiple protein assemblies contain several compounds which together form a functional unit.

Collagen (Fig. 8.23), a fibrous protein found in many connective tissues, consists of three helically coiled linear chains, each of about 1,000 amino acids. Two of these chains (α1) are identical while the third (α2) has a different amino acid composition. The amino acid composition of collagen is 25% glycine and 25% proline and hydroxyproline. In the α-chain glycine is repeated every fourth residue. The triplets gly-pro-pro and gly-pro-Hpro occur frequently.

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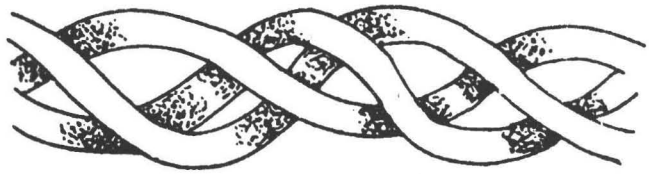

Fig. 8.23. Diagram of the three helically coiled chains of collagen.

Flagella : Microtubules of flagella are made up of *tubulins*. They are mixed dimers (α and β) of two closely related subunits along with small amounts of high molecular weight protein.

Actinomysin complex of muscle : Muscle fibres consist of thin filamants of *actin*, *tropomyosin* and *troponin* which make up the I or light bands (and also extend into the A bands) and thick filaments of *myosin* which make up the A or dark bands.

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9 Enzymes, Coenzymes and Isozymes

9.1 Introduction

Enzymes are proteins which accelerate the rates of the wide variety of chemical reactions which occur in biological systems under thermodynamically unfavourable conditions. A catalyst is a substance which participates in a chemical reaction to enhance its rate without destruction or irreversible modification during the reaction. Enzymes are therefore considered as biological catalysts. The word enzyme from Greek meaning in yeast) was introduced by Kuhne in 1878 to refer to the occurrence in yeast of something responsible for its fermentative activity.

A catalyst influences the rate of a chemical reaction, usually without undergoing any change itself. In this respect an enzyme differs from a normal catalyst. The enzyme may participate in a reaction by combining with the substrate. Ultimately, however, it is set free.

It is considered that, in energetic terms, the reactants do not directly yield products. According to the transition-state theory (based on the work of Eyring) the reaction proceeds through a high-energy state called the transition state. The kinetic energy of the reactants provides the energy for their delivery to the transition state. The energy required is called the activation energy (E_a) which may be evaluated from an arrhenius plot (log reaction rate versus temperature^{-1}). The transition state is a state of maximum energy not an intermediate compound. At this point, the reaction may proceed to form products or revert to initial state reactants. The concentration of the 'activated complex' at the transition state determines the rate of the chemical reaction. Enzymes achieve their reactions at relatively low temperatures by reducing the E_a required for the reaction because of the geometry of their reversible blinding of the reactants. Although E_a is lowered, the net energetics of the reaction remains unaltered.

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The stages of an enzyme-catalysed reaction involving only one reactant can be written as :

$$E + S \rightleftharpoons ES^* \rightleftharpoons ES \rightleftharpoons EX^* \rightleftharpoons EP \rightleftharpoons EP^* \rightleftharpoons P + E$$

where S = reactant called substrate, P = product, ES and EP = enzyme complexes and ES*, EX* and EP* = 'activated complexes' at transition states. For convenience, such reactions are frequently abbreviated to

$$E + S \rightleftharpoons ES \rightleftharpoons P + E$$

where ES = the enzyme-substrate complex.

9.2 Classification and Nomenclature of Enzymes

Enzymes are generally named by adding '-ase' to the root indicating the substrate on which the enzyme acts. Thus *fumarase* catalyses the conversion of fumaric acid to malic acid. The, international Union of Biochemistry Report of 1962 (revised in 1964) contains a scheme for the classification of enzymes. Enzymes have been divided into 6 groups, oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases (synthetases).

(1) *Oxidoreductases* include a large number of enzymes (221 are listed). They bring about the main energy-yielding reactions of living tissue. Oxidoreductases include *oxidases* and *dehydrogenases*. They act by transferring electrons and hydrogen ions.

(2) *Transferases* are concerned with the transfer of a group of atoms from one molecule to another. Oxidoreductases and transferases together represent over half the enzymes known.

(3) *Hydrolases :* Complex molecules undergo cleavage, and the elements of H_2O are added across the bond cleaved by the action of hydrolases.

(4) *Lyases* may work in two ways. A group of atoms may be *removed* from the substrate leaving double bonds, or groups may be *added* to double bonds with hydrolysis, oxidation or reduction. The enzymes act on the following bonds : C—C, C—O, C—N, C—S and C—halide.

(5) *Isomerases* catalyse reactions which bring about intramolecular rearrangement of atoms in substrates.

(6) *Ligases* (*synthetases*) catalyse reactions in which the pyrophosphate bond of ATP is broken down, and linkage takes place between two molecules. These enzymes form the following bonds :

C—O, C—S, C—N and C—C (see Table 9.1)

Table 9.1

THE SIX MAIN DIVISIONS IN ENZYME CLASSIFICATION

No.	*Division*	*Catalytic activity*
1.	Oxidoreductases	Enzymes catalysing oxidoreduction reactions. The substrate is regarded as the hydrogen donor
2.	Transferases	Enzymes transferring a group from one compound to another compound
3.	Hydrolases	Enzymes catalysing the hydrolytic cleavage of C—O, C—N, C—C plus some other bonds
4.	Lyases	Enzymes cleaving C—C, C—O, C—N and other bonds by elimination, leaving double bonds or rings, or conversely adding groups to double bonds
5.	Isomerases	Enzymes catalysing geometric or structural changes within one molecule
6.	Ligases	Enzymes catalysing the joining together of two molecules coupled with the hydrolysis of a pyrophosphate bond in ATP or a similar triphosphate.

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In another system of classification the name of the enzyme is derived from its substrate, *e.g.*, carbohydrases, proteases, dehydrogenases, oxidases, decarboxylases, hydrases, isomerases, transferases, amidases and esterases.

The classification given by the International Union of Bio-chemistry (IUB) is used now. Although this system is complex, yet it is precise, descriptive and informative. The main features of the IUB system for classification of enzymes are as follows :

(1) Reactions and the enzymes that catalyse them have been divided into 6 classes, each with 4-13 subclasses.

(2) The name of enzyme has 2 parts. The first part reveals the substrate or substrates. The second ending in -ase indicates the type of reaction catalysed.

(3) If additional information is needed to classify the reaction, it may follow in parentheses. For example, the enzyme catalysing the following reaction is designated 1.1.1 37 L-malate : NAD oxidoreductase (decarboxylating).

$$\text{L-Malate} + NAD^+ \longrightarrow \text{Pyruvate} + CO_2 + NADH + H^+$$

(4) Each enzyme has been assigned a systemic code number (E.C.). This number characterises the reaction type as to class (*first digit*), subclass (*second digit*) and sub-subclass (*third digit*). The *fourth digit* is for the specific enzyme. For example, an enzyme is assigned a systemic code number such as E.C.2.7.1.1 which denotes class 2 (transferase), subclass 7 (transfer of phosphate), subclass 1 (an alcohol functions as the phosphate acceptor). The final digit represents the enzyme hexokinase or ATP : D-hexose-6 phosphotransferase, an enzyme which catalyses phosphate transfer from ATP to the hydroxyl group on carbon 6 of glucose. In the above nomenclature E.C. indicates enzyme commission number.

There are mainly six classes of enzymes which are described as follows (The name in brackets is the trivial name) :

1. Oxidoreductases

These are the enzymes which catalyse oxidoreductions between 2 substrates S and S'

$$S_{reduced} + S'_{oxidised} = S'_{oxidised} + S'_{reduced}$$

The enzymes including in this class are catalysing oxidoreductions of CH—OH, CH—CH, C = O, CH—NH_2 and CH = NH groups. Representative subclasses of this class of enzymes are as follows :

1.1 Enzymes acting on the CH—OH Group as electron donor

An example is as follows :

1.1.1.1 Alcohol : N A D oxidoreductase (alcohol dehydrogenase) catalyses the following reaction :

Alcohol + NAD^+ = Aldehyde or ketone + NADH + H^+.

1.4 Enzymes acting on the CH—NH_2 group or electron donor. An example is as follows :

1.4.1.3 L-Glutamate : NAD (P) oxidoreductase (deaminating) [Glutamic dehydrogenase of animal liver] catalyses the following reaction : [By NAD (P) it means either NAD^+ or $NADP^+$ which acts as the electron acceptor].

L-Glutamate + H_2O + NAD $(P)^+$ = α-ketoglutarate + NH_4^+ + NAD (P) H + H^+

1.11 Enzymes acting on H_2O_2 as electron acceptor : An example is as follows :

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1.11.1.6. H_2O_2 : H_2O_2 oxidoreductase (catalase) catalyses the following reaction :

$$H_2O_2 + H_2O_2 = O_2 + 2H_2O$$

2. Transferases

This class includes enzymes which catalyse a transfer of a group G (other than hydrogen), between a pair of substrates, S and S'.

$$S - G + S' = S' - G + S$$

Enzymes including in this class are catalysing the transfer of one-carbon groups, aldehyde or ketone residues and acyl, alkyl, glycosyl, phosphorus or sulphur containing groups. Representative subclasses of this class of enzymes are as follows :

2.3 Acyltransferase : An example is as follows :

2.3.1.6 Acetyl-CoA : choline O-acetyltransferase (choline acyltransferase) catalyses the following reaction :

$$\text{Acetyl-CoA} + \text{Choline} = \text{CoA} + \text{O-Acetylcholine}$$

2.7. Enzymes catalysing transfer of phosphorus containing groups : An example is as follows :

2.7.1.1 ATP : D-dexose-6 phosphotransferase (hexokinase) catalyses the following reaction :

$$\text{ATP} + \text{D-Hexose} = \text{ADP} + \text{D-Hexose 6-phosphate}$$

3. Hydrolases

This class includes enzymes which catalyse hydrolysis of ester, ether, peptide, glycosyl, acid anhydride C—C, C—halide or F—F bonds, The subclasses of this class of enzymes are as follows :

3.1 Enzymes acting on ester bonds : An example is as follows :

3.1.1.8 Acylcholine acylhydrolase (pseudocholine sterase) catalyses the following reaction : An acylcholine + H_2O = cooling + An acid.

3.2 Enzymes acting on glycosyl compounds: An example is as follows :

3.2.1.23 β *D-Galactoside galactohydrolase (β-gatactosidase)* catalyses the following reaction.

A β-D-Galactoside + H_2O = An alcohol + D-Galactose

3.4 Enzymes acting on peptide bonds : There are 11 subclasses for making distinction between peptidases and proteases, whether dipeptides or longer peptides are substrates, whether one or more amino acids are removed and whether attack is taking place from the C- or the N-terminal end. Further proteinases are distinguished by their catalytic mechanism as serine, —SH or metalloenzyme proteinases.

3.4.21 Serine proteinases. Examples of these are chymotrypsin, trypsin, plasmin, coagulation factors IX a and XI a.

3.4.23. Carboxyl (acid) proteinases. Examples of these are pepsin A, B and C.

4. Lyases

These include enzymes that catalyse removal of group from substrates by mechanisms other than hydrolysis, leaving double bonds.

$$\begin{array}{l} X \quad X \\ | \quad | \\ C—C = X—Y + C = C \end{array}$$

Examples of this class or enzymes which are acting on C—C, C—O, C—N, C—S and C—halide bonds.

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4.1.2 Aldehyde-lyases : An example is as follows :

4.1.2.7 Ketose-1-phosphate aldehyde-lyase catalyses the following reaction.

A ketose-I-phosphate = Dihydroxy acetone phosphate + An aldehyde

4.2 Carbon-oxygen lyases : An example is as follows :

4.2.1.2 L-Malate hydro-lyase (fumarase) catalyses the following reaction

$$\text{L-Malate} = \text{Fumarate} + H_2O$$

5. Isomerases

These include all enzymes which catalyse interconversion of optical, geometric or positional isomerase. Two subclasses of these enzymes are :

5.2 Cis-Trans isomerases: An example is as follows :

5.2.1.3 All-trans retinene 11-cis trans isomerase (retinene isomerase) catalyses the following reaction :

All *trans* retinene = *11-cis*-retinene.

5.3 Enzymes catalysing interconversion of aldoses and ketoses : An example is as follows :

5.3.1.1 D-Glyceraldehyde-3-phosphate ketolisomerase (triosephosphate isomerase) catalyses the following reaction :

D-Glyceraldehyde-3-phosphate = Dihydroxyacetone phosphate

6. Ligases

These include enzymes which are catalysing the linking together of 2 compounds coupled to the breaking of a pyrophosphate bond in ATP or a similar compound. Examples are enzymes which are catalysing reactions forming C—O, C—S, C—N and C—C bonds. Subclasses of ligases are as follows :

6.2 Enzymes catalysing formation of C—S bonds.

6.3 Enzymes catalysing formation of C—N bonds : An example is as follows :

6.3.1.2 L-Glutamate : ammonia ligase (ADP) (glutamine synthetase) catalyses the following reaction

$$\text{ATP} + \text{L-Glutamate} + NH_4^+ = \text{ADP} + \text{Orthophosphate} + \text{L-Glutamine}$$

6.4 Enzymes catalysing formation of C—C bonds : An example is as follows :

6.4.1.2 Acetyl-CoA : CO_2 ligase (ADP) (acetyl CoA carboxylase) catalyses the following reaction :

$$\text{ATP} + \text{Acetyl—CoA} + CO_2 = \text{ADP} + \text{Pi} + \text{Malonyl-CoA}$$

9.3 Physico-Chemical Nature of Enzymes

All enzymes are proteins. Proteins are high molecular weight macromolecules. The molecular weights of enzymes range from about zeros to many million. An enzyme may consist of a single polypeptide chain, *e.g.,* beef ribonuclease, or an aggregate of polypeptide chains. The polypeptide chain is made up of a number of amino acid units linked by peptide bonds. The sequence and number of the 20 amino acids which make up enzymes vary in different enzymes. The sequence is specific for a particular enzyme and determines the properties of the enzyme.

The amino acid sequence and the three-dimensional structure have been determined for the enzymes *egg-white lysozyme, ribonuclease, carboxypeptidase, chymotrypsin and papain. Lysozyme*

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hydrolyses links between amino sugars, and is found in saliva and egg white. *Ribonuclease* degrades RNA into small fragments. *Chymotrypsin and trypsin* are proteolytic enzymes found in the pancreas. *Chymotrypsinogen,* the inactive precursor of chymotrypsin, consists of a single polypeptide chain. Removal of two dipeptides results in the active enzyme chymotrypsin, which consists of three polypeptide chains. Lysozyme, ribonuclease, carboxypeptidase, chymotrypsinogen and papain each consists of a single polypeptide chain which is highly folded into a spherical shape. Trypsinogen consists of two polypeptide chains, and chymotrypsin of three chains. The number of amino acid residues is 33 in carbonic anyhydrase, 124 in ribonuclease, 129 in egg-white lysozyme and 249 in chymotrypsinogen and trypsinogen.

The polypeptide chain has an *amino* (NH_2) terminal and a *carboxy* (COOH) terminal. Biosynthesis of the enzyme begins at the amino terminal.

The different parts of the polypeptide chain are linked by disulphde (-S S-) bridges, which are most commonly found between two *cystein* amino acids. Disulphide bridges may be within a plypeptide chain (*interchain*) or may connect two polypeptide chains (*interchain*). Egg-white lysozyme and ribonuclease have four disulphide bridges, chymotrysinogen has five and trypsinogen six.

Generally the enzymes are made up of two portions called a coenzyme and an aponenzyme.

Coenzyme (prosthetic group) + apoenzyme ⟶ (holo enzyme)

Both portions become enzymatically inactive when they are separated from each other. Specificity of an enzyme to the substrate is ascribed to apoenzyme portion. Chemical analyses reveal that this portion is a protein which is further composed of 1-amino acid units. Enzymes either exist as single units or as aggregates of several subunits. Each subunit may be having an active centre at which substrate molecules bind during reaction.

9.4 Enzyme Kinetics

I. Energy of activation : Enzymes increase the speed of a chemical reaction. They lower the energy of activation of a reaction, thus enabling it to occur at ordinary physiological temperatures. When reactions proceed from one direction to another they have to overcome an energy barrier

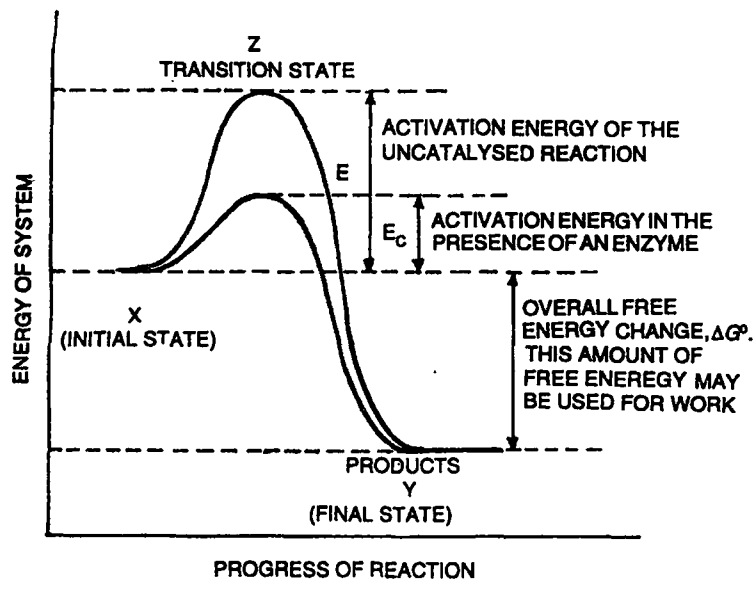

Fig. 9.1. Energy profile for uncatalysed and enzymic reactions.

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called the *activation energy*. Normally only a small part of the total number of molecules in a compound contain enough energy to react. Application of heat enables a larger proportion of molecules to overcome the activation energy barrier. However, living systems are relatively isothermal, *i.e.,* they do not have large temperature differences. They therefore employ enzymes to activate the molecules of the reacting compounds. When the enzyme combines with the substrate to form the enzyme-substrate complex the energy level of the substrate is raised, and it reacts faster. Enzymes increase the speed of a chemical reaction thousands of times by bringing about mutual contact of reacting compounds. The coming together of these compounds is no longer a matter of chance, but becomes a certainty.

In Fig. 9.1, the chemical compound X is in a metastable state and it requires E as energy of activation to convert the reactant in a new activated state Z. The reaction then becomes spontaneous, converting Z into Y and energy E is liberated. A further amount of energy is released according to the difference in enthalpy (ΔH) of X and Y. Thus enzymes increase the rate of conversion of X into Y with a lower energy of activation. For example, the energy of activation for the decomposition of H_2O_2 is 18 K cal $mole^{-1}$, while in the presence of enzyme catalyses it is only 6.4 K cal $mole^{-1}$. Thus, the energy of activation in this case becomes about one-third in the presence of enzyme.

II. Steady-State Enzymes Kinetics (Michaelis constant) : A basic theory of enzyme action was proposed in 1913 when Michaelis and Menten developed a mathematical expression to rationalise the hyperbolic plot of V_0 as a function of [S]. The Michaelis-Menten equation aims to describe the interrelationship between the parameters pertaining to an enzymic reaction. This accomplishment was based on two assumptions :

(*i*) the enzyme-substrate complex (ES) is in equilibrium with free enzyme and substrate in solution, *i.e.,* $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES$ and

(*ii*) the formation of this complex is essential for product formation, *i.e.,* $ES \rightarrow P + E$.

The current derivation of their equation incorporates a further assumption, introduced by Briggs and Haldane in 1925.

(*i*) Consider the formation of ES

$$\therefore \frac{d[ES]}{dt} = k_1 [E] [S]$$

As the steady-state assumption is justifiable only where

$[S] > 1000 \times [E]$

(*a*) differentiate between free and combined enzyme and

(*b*) ignore ES formation by the reverse direction, $ES \xrightarrow{k_{-1}} E + S$ and $ES \xrightarrow{k_2} P + E$ because the reverse reaction is negligible initially

$$\therefore \frac{d[ES]}{dt} = k_1 ([E_t] - [ES]) [S]$$ where E_t = total enzyme (free and combined forms)

(*ii*) Consider the breakdown of ES, *i.e.,* in forward and reverse directions :

$$\frac{-d[ES]}{dt} = k_{-1} [ES] + k_2 [ES]$$

(*iii*) Consider ES in a steady-state in which [ES] is constant : *i.e.,* rate of ES formation = rate of ES breakdown

$$\therefore k_1 ([E_t] - [ES]) [S] = k_{-1} [KS] + k_2 [ES]$$

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(*iv*) Group the constants :

$$\frac{([E_t]-[ES])\,[S]}{[ES]} = \frac{k_{-1}+k_2}{k_1}$$

(*v*) The rate constant grouping is denoted by K_m, the Michaelis constant.

(*vi*) From $\frac{([E_t]-[ES])\,[S]}{[ES]} = K_m$, solve for [ES]}

$$\therefore \frac{[E_t][S]}{[ES]} - \frac{[ES][S]}{[ES]} = K_m$$

$$\therefore \frac{[E_t][S]}{[ES]} - [S] = K_m$$

$$\therefore \frac{[E_t][S]}{[ES]} = K_m + [S]$$

$$\therefore [ES] = \frac{[E_t][S]}{K_m + [S]}$$

Since, $V_0 = k_2[ES]$, substitute for [ES]

$$\therefore V_0 = \frac{k_2[E_t][S]}{K_m + [S]}$$

Also, [S] is so high that essentially all the enzyme is present as ES, *i.e.,* the enzyme is saturated and the maximum velocity, V, is achievable, but

$$V = k_2[E_t]$$

$$V_0 = \frac{V[S]}{K_m + [S]}$$

This is the Michaelis-Menten equation

The Michaelis-Menten equation relates the components of an enzymic reaction, [S] and [E], to velocity, initial and maximum, through a rate constant, called K_m, the Michaelis constant, where

$$K_m = \frac{\text{rate of breakdown of the ES complex}}{\text{rate of formation of the ES complex}}$$

V or (V_{max}) is defined as the maximum velocity of an enzymic reaction. K_m is defined as the substrate concentration at which an enzyme demonstrates 50% of its maximum velocity.

We have already proved that

$$V_0 = \frac{V[S]}{Km + [S]}$$

when

$$V_0 = \frac{1}{2}V$$

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then

$$\frac{V}{2} = \frac{V[S]}{K_m + [S]}$$

Divide by V :

$$\frac{1}{2} = \frac{[S]}{K_m + [S]}$$

Cross multiply :

$$K_m + [S] = 2[S]$$

$$K_m = [S]$$

K_m is therefore the [S] at which half of the active sites of the enzymes are occupied. The K_m is a constant characteristic of an enzyme for its conversion of a substrate. V is dependent upon [E] which is thereby represented in the equation. Modern enzyme kinetics is founded upon this equation which allows the rate of reaction at any [S] to be calculated, if K_m and V are known. This is of importance in understanding the behaviour of metabolic pathways. The smaller the value of K_m the greater the enzyme's affinity for the substrate and the quicker the reaction : The largest K_m represents the rate-limiting reaction in a metabolic pathway.

To estimate K_m and *V*, the initial rate a measured at several different substrate concentrations. However, the hyperbolic plot of initial velocity as a function of [S] is inadequate to determine V accurately since at an [S] of $10 \times K_m$ only approximately 90% V is achieved [Figure 9.2 (*a*)]. At greater concentrations, additional problems such as substrate insolubility, formation of non-productive enzyme-substrate complexes through substrate inhibition mechanisms and salt effects may be encountered. To obtain an accurate estimation of V and hence K_m, enzymologists rearranged the Michaelis-Menten equation to produce linear plotting methods, *e.g.*, Lineweaver-Burk, Eadie-Hof-stee [Figure 9.2 (*b*) and (*c*)] and other plots.

We have already proved

Michaelis-Menten equation : $$V_0 = \frac{V[S]}{K_m + [S]}$$

Take reciprocals of both sides : $$\frac{1}{V_0} = \frac{K_m + [S]}{V[S]}$$

Rearrange : $$\frac{1}{V_0} = \frac{K_m}{V[S]} + \frac{[S]}{V[S]}$$

Reduce to the Lineweaver-Burk equation : $$\frac{1}{V_0} = \frac{K_m}{V} \cdot \frac{1}{[S]} + \frac{1}{V}$$

The line-weaver-Burk plot is a popular method although the use of reciprocals results in points nearest to the origin representing the highest rates and [S] values and emphasizes less precise measurements at low [S] with less accent on more accurate higher rates. The Eadie-Hofstee plot

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aims to improve the accuracy of V and K_m data by improvements in the weighting and separation of points and the exactness of the line but suffers from compounding errors by not retaining the independence of the variables. Computerised line-fitting techniques have been applied to the plotting of kinetic data. Variations from the characteristic plot may indicate contamination by activators, inhibitors or impurities. The properties of enzymes catalysing two-substrate reactions can be studied by varying the concentration of each substrate in the presence of a saturating concentration of the other substrate and employing standard graphical procedures.

We know $$V_0 = \frac{V[S]}{K_m + [S]}$$

when $$V_0 = \frac{1}{2}V$$

then $$\frac{V}{2} = \frac{V[S]}{K_m + [S]}$$

Divide by V : $$\frac{1}{2} = \frac{[S]}{K_m + [S]}$$

Cross multiply : $$K_m + [S] = 2[S]$$

$$K_m = [S]$$

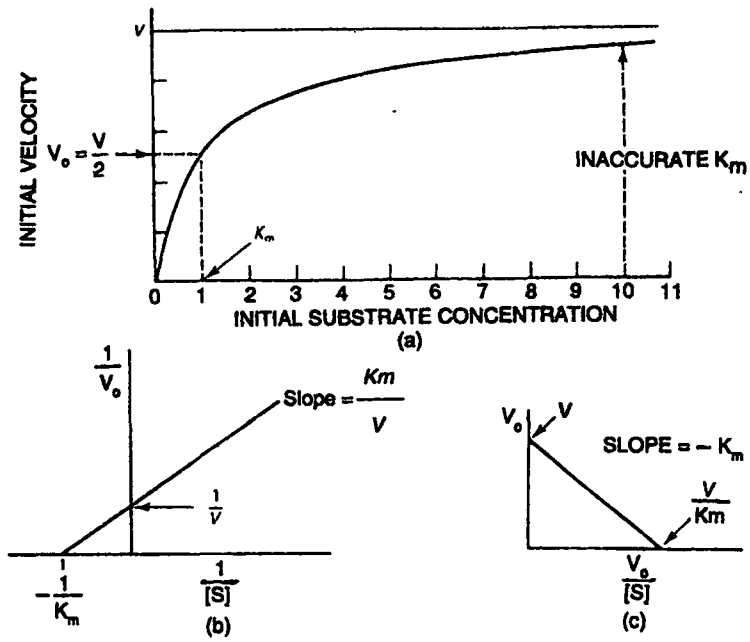

Fig. 9.2. Graphical procedures for the estimation of V and K_m (*a*) Plot of initial velocity versus substrate concentration. (*b*) Lineweaver-Burk plot. (*c*) Eadie Hofstee plot.

The values of Michaelis constant for most of the enzymes have been found to vary from 10^{-2} and 10^{-5} M substrate concentration. The value of Michaelis constant has been found to be inversely

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proportional to the enzyme activity. A large K_m implises that a high substrate concentration is needed to get half of the maximum rate. In other words, it means that enzyme has lower affinity for the substrate.

9.5 Activity of Enzymes

The activity of enzymes can be expressed in a number of ways :

1. The recommended unit of enzyme activity is the katal, which is the amount of activity that converts 1 mol of substrate s^{-1}. Frequently, the activities are expressed in microkatals (μkat), nanokatals (nkat) etc. corresponding to reaction rates of μmol, nmol s^{-1} respectively.
2. An older term, the International Enzyme Unit (IU), is defined as the amount of enzyme which will catalyse the transformation of 1 μmol of substrate min^{-1} under standard conditions.

Other related terms are :

(i) The specific activity of an enzyme preparation is kat kg^{-1} of protein or IU mg^{-1} of protein.

(ii) The molar activity is kat mol^{-1} of enzyme.

(iii) The turnover number of an enzyme is the number of molecules of substrate transformed per active site of the enzyme min^{-1}.

9.6 Factors Affecting Enzyme Activity

General : In an enzyme-substrate reaction mixture at a constant temperature, the reaction velocity decreases as a function of time [Figure 9.3]. This decline may occur for numerous reasons :

(i) The approach to an equilibrium and the associated influence of the reverse reaction.

(ii) The depletion of the substrate leads to the reduced occupancy of active sites.

(iii) The products of the reaction may inhibit the enzyme or change the pH if the medium is inadequately buffered.

(iv) The enzyme may undergo some progressive inactivation at the temperature or pH of the reaction due to instability.

Enzymes are therefore studied within their initial velocity (V) range, *i.e.*, at the initial stage before these factors exert an influence on the reaction. The initial velocity may be determined from the slope of a tangent to the curve at time zero (V_0).

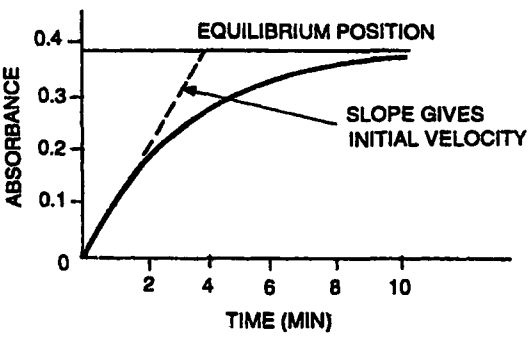

Fig. 9.3. Progress curve of typical enzyme-catalysed reaction.

1. Concentration of enzymes : Increase in enzyme concentration has been found to increase enzyme activity. This is because of the provision of additional catalytic sites to which the substrate may bind with concomitant rate enhancement. When an excess of substrate is present, increasing the enzyme concentration by two times generally doubles the rate of formation of the product. With the given concentration of the enzyme also, a point may be reached where all the substrate molecules are bound to the enzyme and further increase in enzyme does not have an effect on the formation of the product (Fig. 9.4).

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2. Concentration of substrates : The concentration of substrate also influences the initial velocity but not in a simple manner [Fig. 9.5 (*b*)]. At a constant [E], the hyperbolic plot obtained with different initial substrate concentrations shows that the rate is initially proportional to [S], *i.e.*, first order with respect to substrate. (In the orders of chemical reactions, the reaction is of first order when its rate is proportional to the first power of the concentration of just one reactant.) At extremely high substrate concentrations, the reaction rate approaches a constant rate. This is the maximum velocity (V or V_{max}) attainable for this particular [E]. The available active sites of all the enzyme molecule are occupied by the substrate; the enzyme is saturated. To increase the rate additional active sites must be made available by the addition of more enzyme. The reaction rate at V is independent of [S] and is of zero order with respect to substrate. Between the extremities, the reaction is a mixture of zero- and first-order kinetics. This behaviour of an enzyme led to the concept of an enzyme-substrate complex intermediate and the underpinning of modern enzyme kinetics by the Michaelis-Menten equation.

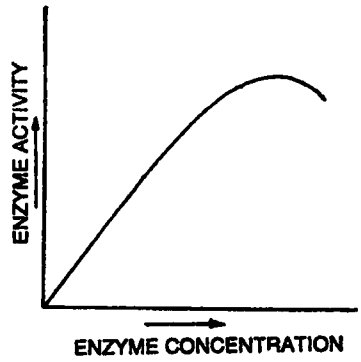

Fig. 9.4. Effect of enzyme concentration on enzyme activity.

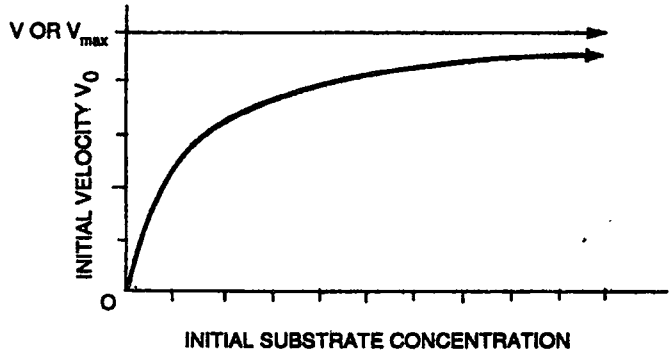

Fig. 9.5. Effect of substrate concentration on reaction rate when enzyme and substrate are incubated for a constant time.

3. Enzyme inhibition (Enzyme inhibitors) : In addition to substrate concentration, two groups of compounds alter the rate of an enzymic reaction by specific mechanisms. Activators are compounds which combine with an enzyme or enzyme-substrate complex to effect an increase in activity without being modified by the enzyme. Inhibitors are compounds which decrease the rate of an enzyme-catalysed reaction.

Inhibitors are divided into two categories : irreversible and reversible inhibitors.

(a) Irreversible inhibition : Irreversible inhibition involves the covalent bonding of the inhibitor to a functional group at the active site or elsewhere on the enzyme. Because progressively declining, irreversible inhibition cannot be analysed by Michaelis-Menten kinetics. This type of inhibition is frequently used to obtain information regarding the functional amino acids at the active site of the enzyme. Irreversible inhibitors have been exploited to provide pesticides, *e.g.*, parathion is an active inhibitor of insect acetylcholinesterase upon which normal propagation of nervous

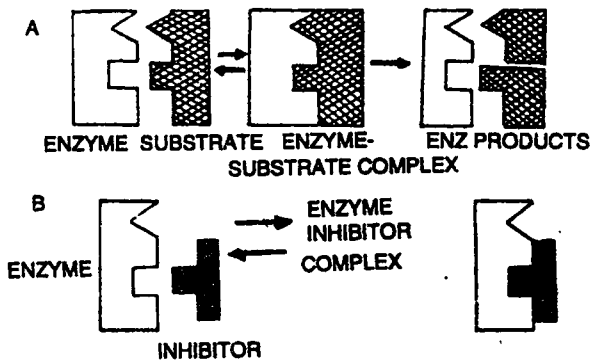

Fig. 9.6. (A) Enzyme action. (B) Inhibition.

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impulses relies. Suicide inhibition is a form of irreversible inhibition in which the substrate in the first catalytic cycle is converted into a chemically reactive product which remains bound to the active site through covalent bonding. The enzyme is rendered permanently inactive. Such inhibitors have potential as drugs.

(b) Reversible inhibition : Reversible inhibitors are not covalently bound to the enzyme. Reversible inhibition is characterised by an equilibrium between free and inhibitor-bound enzymic forms. Therefore, steady-state kinetics may be applied to the analysis of these inhibitions. The inhibitor may be removed from the enzyme inhibitor (EI) complex by simple methods to furnish active enzymes. Of the various forms of reversible inhibition which have been identified through kinetic studies, competitive, non-competitive and uncompetitive inhibition will be considered here.

Competitive inhibitors are inhibitors which have an effect on the K_m but not on the V of an enzyme-catalysed reaction. The V is unchanged because the number of functional active sites is not altered but a greater substrate concentration is required to achieve the maximum utilization of the sites. Consequently, the K_m for the substrate increases. Competitive inhibition may be overcome by the addition of more substrate to the enzyme reaction mixture. Competitive inhibitors often bear a structural similarity to the substrate and complete with the substrate for the active sites of the enzyme, *i.e.*, they are isosteric. However, competitive inhibitors are not necessarily structually analogous to the substrate, *e.g.*, salicylate inhibition of 3—phosphoglycerate kinase, and may bind to a site distinct from the active site, *e.g.*, L-isoleucine inhibition of threonine deaminance from *Escherichia coli*. The classical example of competitive inhibition is the action of malonate on succinate dehydrogenase (Fig. 9.7) which advances the elucidation of the tricarboxylate cycle. Inhibitors of this reaction achieve their effects by ionic bonding to the contact amino acid residues.

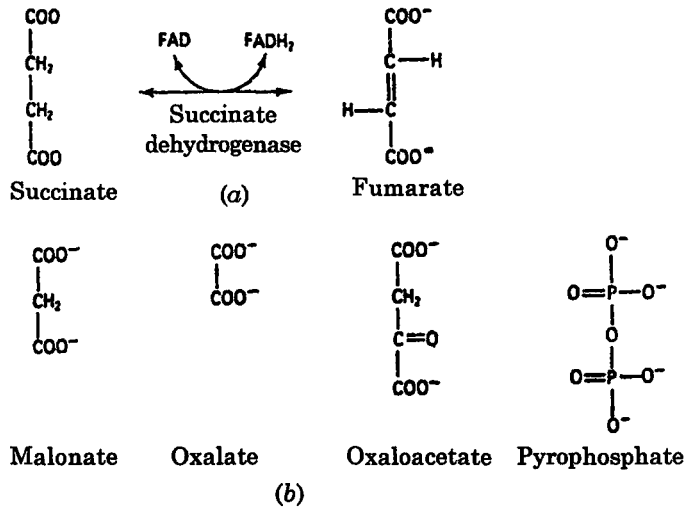

Fig. 9.7. Competitive inhibition of succinate dehydrogenase. (*a*) The reaction. (*b*) Some inhibitors.

Non-Competitive Inhibition

Non-Competitive inhibition is characterised by a change in V but not K_m and is not reversed by addition of substrate. This type of inhibition occurs when an inhibitor binds to a site other than the active site on the free enzyme or enzyme-substrate complex. Non-competitive inhibitors interfere with either the formation of ES complex or its breakdown to yield product. Examples include heavy metal (*e.g.*, mercury, lead) ions which bind to strategically positioned sulphydryl groups and modulate the enzyme's conformation.

Uncompetitive Inhibition

Uncompetitive inhibitors bind only to a formed ES complex. This type of inhibition, characterised by equal effects on both V and K_m is rare in one-substrate reactions but may occur as a type of product inhibition in reactions with multiple substrates and products. Figure 9.8

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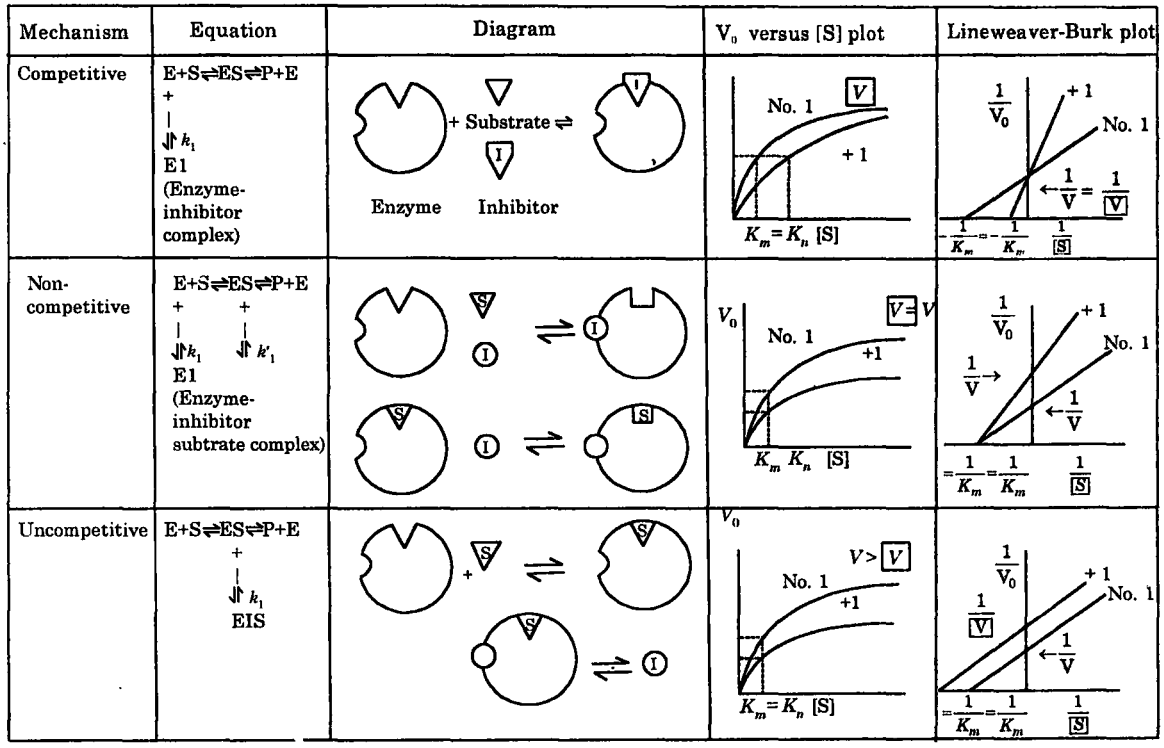

Fig. 9.8. Mechanisms of reversible inhibition.

illustrates mechanistic and plot differences between the discussed inhibitions. Table 9.1 summarizes the effect of inhibitors on Lineweaver-Burk plot parameters. Graphical methods are available for the estimation of K_i, the inhibition constant. In competitive inhibition, for example

$$K_i = \frac{[E]\,[I]}{[EI]\}}$$

Table 9.2

INTERCEPTS ON AXES OF A LINEWEAVER-BURK PLOT IN THE ABSENCE AND PRESENCE OF INHIBITORS

	Intercept on 1/n axis	*Intercept on 1/[S] axis*	*Slope*
No inhibitor	$\frac{1}{V}$	$\frac{-1}{K_m}$	$\frac{K_m}{V}$
Competitive inhibitor	$\frac{1}{V}$	$\frac{-1}{K_m\left(1+\frac{[I]}{K_i}\right)}$	$\frac{K_m}{V}\left(1+\frac{[I]}{K_i}\right)$
Non-competitive* inhibitor	$\frac{1}{V}\left(1+\frac{[I]}{K_i}\right)$	$\frac{-1}{K_m}$	$\frac{K_m}{V}\left(1+\frac{[I]}{K_i}\right)$
Uncompetitive inhibitor	$\frac{1}{V}\left(1+\frac{[I]}{K_i}\right)$	$\frac{-1}{K_m\left(1+\frac{[I]}{K_i}\right)}$	$\frac{K_m}{V}$

*K may be K'_i or K_1 depending on mechanism.

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4. Effect of pH : Each enzyme exhibits maximum activity at a characteristic pH called its optimum pH (Figure 9.9). The bell-shaped plot illustrates that limited departures from this pH leads to a reduced enzymic performance due to changes in the ionization of contact and catalytic amino acid residues. pH changes also modify ionic substrates. If a COO^- group of an asparate is required to bind a positively charged substrate, protonation would reduce the force of attraction and decrease the affinity of the enzyme for the substrate although it becomes more positively charged. The stability of the enzyme may be compromised by pH-induced conformational changes with loss of activity. This may occur either on both sides of the optimum pH or in combination with the above effects.

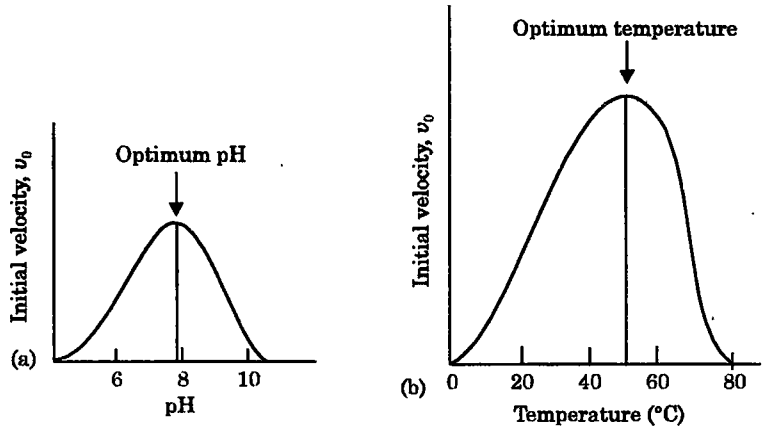

Fig. 9.9. Effects of (*a*) pH and (*b*) temperature on reaction rate when enzyme and substrate are incubated for a constant time.

5. Effect of temperature : The temperature at which an enzymic reaction is measured profoundly affects V_0 [Figure 9.9 (*b*)]. Enzymic reactions occur slowly at 0°C because of the lower level of molecular kinetic energy which limits substrate-enzyme collisions and the attainment of the transition state. Increasing temperature promotes both these events. The approximately two-fold rate enhancement per 10°C rise reflects the activation energy requirement to achieve the transition state. At higher temperatures, product formation declines due to conformational changes in the enzyme by thermal denaturation which reduces the effective [E]. The maximum rate of substrate conversion into product is time dependent. As the duration of incubation is extended, the plot in Figure 9.9 (*b*) is displaced to the left.

6. Effect of ions : Many enzymes only become active in the presence of a cation such as Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Na^+ or K^+. In some cases, the cations get loosely bound to the enzyme, while in other cases they get bound to the substrate. Anions have also been found to increase the enzyme activity. For example, chloride enhances the activity of salivary amylase. Concentration of ions also influences the enzyme activity.

7. Redox potential : Enzymes have been found to be sensitive to the redox potential of the cell also. Oxidising and reducing enzymes also take part in changing the redox component of a cell and hence they get affected by the redox potential. Some enzymes are affected by redox potential due to the presence of readily oxidisable—SH group in their molecule.

9.7 Mechanism of Enzyme Actions

We have seen that the action of enzymes is highly specific. What kind of mechanism can account for such specificity? It was suggested by Arrhenius about 100 years ago that catalysts

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speed up reactions by combining with the substrate to form some kind of intermediate compound. In an enzyme-catalyzed reaction, the intermediate is the enzyme-substrate complex.

To account for the high specificity of most enzyme-catalysed reactions, a number of models have been proposed. Some of these are described as follows :

The simplest and most frequently quoted is the lock-and-key model (Fig. 9.10). This model assumes that the enzyme is a rigid three-dimensional body. The surface that contains the active site has restricted opening into which only one kind of substrate can fit, just as only the proper key can fit exactly into a lock.

According to the lock-and-key mechanism, an enzyme molecule has its own particular shape because that shape is necessary to maintain the active site in exactly the geometric alignment required for that particular reaction. An enzyme molecule is very large (typically 100 to 200 amino acid residues), but the active site is usually composed of only two or few amino acid residues, which may well be located at different places in the chain. The other amino acids—those not part of the active site—are located in the sequence in which we find them because that is the sequence that causes the whole molecule to fold up in exactly the required way.

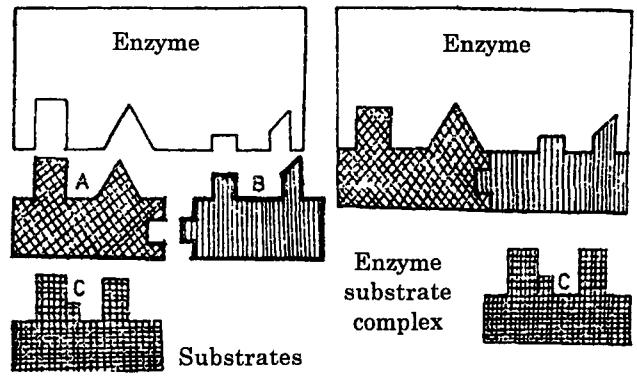

Fig. 9.10. Diagram illustrating how only particular substrates react with an enzyme.

For example, substrates A and B will fit into the enzyme E but not substrate C (Fig. 9.10). This is referred to as a 'lock-and-key' mechanism. Thus reactions involving A and B will be speeded up but, not reactions involving C.

The *enzyme-enters into a chemical combination with the substrate* to form an *enzyme-substrate complex* (Michaelis-Menton hypothesis).

$$E + S \longrightarrow ES$$

The enzyme substrate complexed then breaks down to give the products of reaction. The enzyme is released and can be used over and over again.

$$ES \longrightarrow E + \text{Products}$$

The lock-and-key model explains the action of many enzymes. But of other enzymes, there is evidence that this model is too restrictive. Enzyme molecules are in a dynamic state, not a static one. There are constant motions within them, so that the active site has some flexibility.

Active Site

An enzyme has a distinct cavity or cleft in which the substrate is bound. The cleft contains an *active centre* in which the amino acids are grouped together in such a way as to enable them to combine with the substrate (Fig. 9.11). The reactive amino acids may lie widely separated in the polypeptide chain. The chain, however, undergoes folding in such a manner that the reactive amino acids come together in the active site.

It is believed that when the substrate molecule binds to the active site, its parts are held together in such a way so as to cause distortion of the chemical bonds, *i.e.*, the bonds are weakened.

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This distortion of the chemical bonds of the substrate increases its reactivity, and thus speeds up the rate of the reaction. The products of the reaction are released because they are less firmly bound. The mechanism suggested above has been called the *strain model* of enzyme catalysis.

Another model, called the *rack model*, supposes that the conformational change in the substrate after binding leads to *increased* distortion of the substrate molecule. This bending after binding makes the substrate more reactive.

Another way in which the reactive site is believed to speed up a reaction is by *excluding water molecules* of the solvent from the site of reaction (Fig. 9.12). The tight fit of the substrate in the active site of the enzyme molecule does not permit water molecules at the site of reaction. It is known that in the case of certain compounds exclusion of water molecules greatly affects the rate of reaction.

Induced fit model : This model was given by Koshland (1966). In the Fischer model, *i.e., lack-and-key* mechanism, the catalytic site is presumed to be preshaped to fit the substrate. In the induced fit theory, the substrate induces a conformational change in the enzyme. This aligns amino acid residues or the other groups on the enzyme in the correct spatial orientation for substrate binding, catalysis or both. At the same time, the other amino acid residues may get buried in the interior of the enzyme. This is depicted in Fig. 9.13.

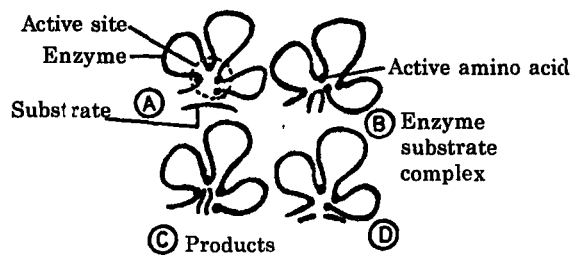

Fig. 9.11. Diagramatic representation of an enzyme and its active site.
(A) Enzyme with its reactive site consisting of four reactive amino acids.
(B) Substrate binds to enzyme to form enzyme-substrate complex.
(C) Reaction occurs in which the substrate is broken down into its products.
(D) Products removed from active site.

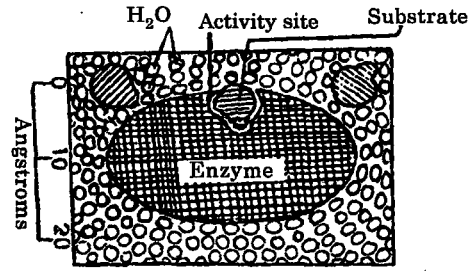

Fig. 9.12. Diagram showing how the formation of an enzyme-substrate complex excludes water molecules from the reactive site of the enzyme. Approximately to scale.

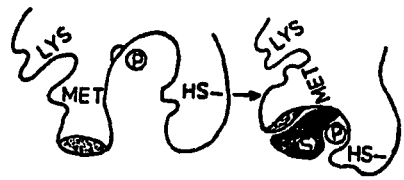

Fig. 9.13. Representation of an induced fit by a conformational change in the protein structure.

In Fig. 9.13, in the absence of substrate, the catalytic and the substrate-binding groups are several bond distances removed from one another. When the substrate approaches, there occurs a conformational change in the enzyme protein, aligning the groups correctly for substrate binding and for catalysis. At the same time there also occurs a change in the spatial orientations of the other regions.

The main evidence in favour of induced fit model comes from demonstration of conformational changes during substrate binding and catalysis with creatine kinase, phosphoglucomutase, and several other enzymes. Upto this time, it could not be estabilshed about the exact sequence of events in a substrate induced conformational change. There may be several possibilities which

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are depicted in Fig. 9.14.

Even if one knows the complete primary structure of an enzyme, it is not very easy to decide exactly which residues exactly constitute the catalytic site.

Both the lock-and-key and the induced-fit model explain the phenomenon of competitive inhibition. The inhibitor molecule fits into the active site cavity in the same way the substrate does preventing the substrate from entering. The result is that whatever reaction is supposed to take place on the substrate does not take place.

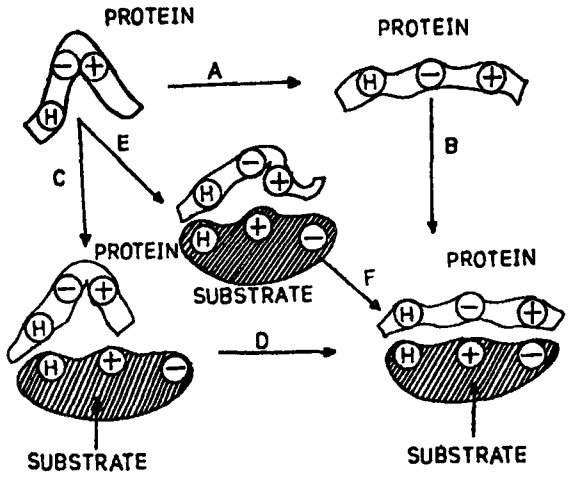

Fig. 9.14. Representation of alternative reaction paths for a substrate-induced conformational change.

Many cases of non-competitive inhibition can also be explained by either model. In this case, the inhibitor does not bind to the active site but to a different part of the enzyme. Nevertheless, the binding causes a change in the three-dimensional shape of the molecule and this so alters the shape of the active site (the lock) that the substrate (the key) can no longer fit.

If we compare enzyme activity in the presence and absence of an inhibitor, we can tell whether competitive or non-competitive inhibition is taking place (Fig. 9.11). The maximum reaction rate is the same without an inhibitor and in the presence of competitive inhibitor. The only difference is that this maximum rate is achieved at a low substrate concentration with no inhibitor but at a high substrate concentration when an inhibitor is present. This is the true sign of competitive inhibition because here the substrate and the inhibitor are competing for the same active site. If the substrate concentration is sufficiently increased, the inhibitor will be displaced from the active site by Le Chatelier's principle.

If, on the other hand, the inhibitor is non-competitive, it cannot be displaced by addition of excess substrate, since it is bound to a different site. In this case the enzyme cannot be restored to its maximum activity, and the maximum rate of the reaction is lower than it would be in the absence of the inhibitor.

The perception of the active site as either a rigid (lock-and-key model) or a partly flexible template (induced-fit model) is an oversimplification. Not only is the geometry of the active site important, but so are the specific interactions that take place between enzyme surface and substrate. To illustrate, we take a closer look at the active site of the enzyme pyruvate kinase (Fig. 9.16). This enzyme catalyzes the removal of the phosphate group from phosphoenol pyruvate (PEP), an important step in glycolysis.

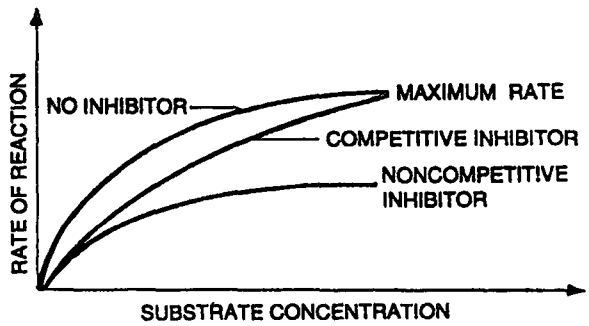

Fig. 9.15. Enzyme kinetics in the presence and absence of inhibitors.

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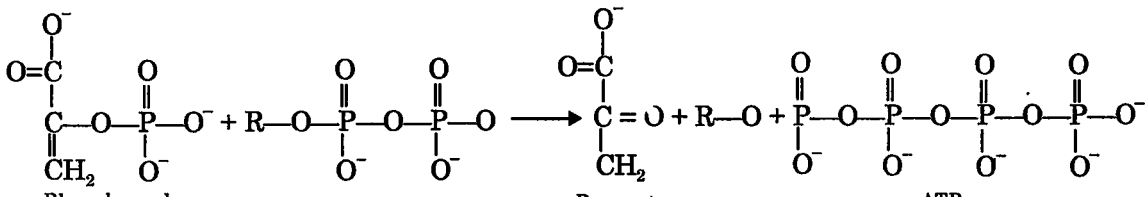

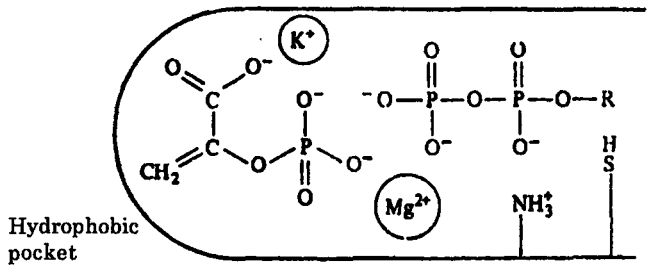

Fig. 9.16. The active site and the substrates of pyruvate kinase.

The active site of the enzyme binds both substrates, PEP and ADP. The enzyme has two cofactors K^+ and Mg^{2+}. The K^+ binds the carboxyl group of the PEP, and the Mg^{2+}. The K^+ binds the carboxyl group of the PEP, and the Mg^{2+} anchors two phosphate groups, one from the PEP, and the Mg^{2+} anchors two phosphate groups, one from the PEP and one from the ADP. Other side chains of the apoenzyme bind the rest of the ADP into the active site. All these acids are in the form of their ions. There is also hydrophobic area on the enzyme that binds the nonpolar—CH_2 unit.

9.8 Coenzymes

Definition : All enzymes are protein molecules. Some enzymes are simple proteins whereas others are conjugated proteins. The non-protein part of a conjugated protein or enzyme is known as prosthetic group. It is observed that prosthetic group is necessary for protein to act as an enzyme and is generally termed as *coenzyme,* the protein part of a conjugated protein as *apoenzyme* and the intact molecule or conjugated protein or *holoenzyme.* Thus,

$$\text{Holoenzyme} \rightleftharpoons \text{Apoenzyme} + \text{Coenzyme}$$

Thus, the *coenzyme may be defined as a non-proteinous substance necessary for the activity of the enzyme.*

Only when enzyme and coenzyme are present together, catalysis will occur.

It is often difficult to distinguish between what is meant by the terms "coenzymes", "prosthetic group" and "activators." Perhaps it is best to reserve the "coenzyme" for the organic fragments that are not normally isolated with the apoenzyme. A "prosthetic group" would then represent a firmly attached coenzyme such as iron protoporphyrin and "activator" might be reserved for metals, non-specific reducing agents, and so forth. At any rate, all these substances are generally of low molecular weights and are dialyzable and heat stable.

Coenzymes are organic molecules of a size intermediate between the small-molecule intermediary metaboltes, which serve as the substrates of enzymatic reactions, and the macromolecular proteins. A coenzyme is an easily dissociable portion (sometimes called the prosthetic portion or group) attached to the protein component (apoenzyme) to form the complete, enzymatically active, conjugated protein (holoenzyme). Each coenzyme (or cofactor) acts usually as acceptor or donor of some specific type of atom or group of atoms to be removed from or added to a small molecule substrate in a reaction catalysed by the hole-enzyme. For example, the folic acid coenzymes accept or donate single carbon units (at various states of oxidation) in a considerable number of enzymatic reactions grouped together as single carbon unit metabolism.

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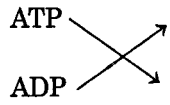
It is often helpful to regard the coenzyme a second substrate, *i.e.*, a cosubstrate. This is attributed to two reasons :

(*i*) The chemical changes in the coenzyme exactly counterbalance those taking place in the substrate. An example is in transphosphorylation reactions involved in the metabolism of sugars for every molecule of sugar phosphorylated, one molecule of ATP is dephosphorylated and converted to AD.



(*ii*) The second reason for the importance of co-enzymes is that their reactions may be of physiological importance. For example, the importance of ability of muscle to convert anerobically pyruvate to lactate resides neither in pyruvate nor in lactate themselves but the reaction merely serves to convert *NADH* to NAD^+.

Table 9.3

CLASSIFICATION OF MAJOR COENZYMES

Class	*Abbreviation*	*Corresponding vitamin*	*Group transferred*
1. Hydrogen transferring coenzymes			
(*i*) Nicotinamide adenine dinucleotide	NAD^+/NADP	Nicotinamide	Hydrogen, electrons
(*ii*) Flavin mononucleotide	FMN	Riboflavin	Hydrogen, electrons
(*iii*) Flavin adenine dinucleotide	FAD	Riboflavin	Hydrogen, electrons
(*iv*) Ubiquinone	Q	Riboflavin	Hydrogen, electrons
(*v*) Lipoic acid	Lip (S_2)	–	and acyl group
(*vi*) Cytochromes	–	–	Electrons
2. Group transferring coenzymes			
(*i*) Adenosine triphosphate	ATP	–	Phosphate, AMP
(*ii*) Adenosyl methionine	–	Methionine	Methyl group
(*iii*) Phospho adenyl sulfate	PAPS	–	Sulfate group
(*iv*) Pyridoxal phosphate	PALP	Pyridoxine	Amino group
(*v*) Cytidine diphosphate	CDP	–	Phosphoryl choline
(*vi*) Uridinediphosphate	UDP	–	Sugar uronic acid
(*vii*) Tetrahydrofolate	COF	Folate	Formyl group
(*viii*) *Biotin*	–	Biotin	Carboxyl group
(*ix*) *Coenzyme A*	CoA	Panthothenate	Acyl group
(*x*) Thiamine pyrophosphate	TPP	Thiamine	C^2-aldehyde group
(*xi*) Glutathione	–	–	Addition of thiol group
3. Isomerase and lyase coenzymes			
(*i*) Urdinediphosphate	UDP	–	Sugar isomerisation
(*ii*) Pyridoxal phosphate	PALP	–	Decarboxylaton
(*iii*) Thiamine pyrophosphate	TPP	Pyridoxine	Decarboxylation
(*iv*) B_{12} coenzyme	–	Thiamine	Carboxyl displacement
(*v*) Glutathione	–	Cobalamin	Isomerization of olefins

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Without NAD^+, glycolysis cannot occur and anaerobic ATP synthesis ceases and the hence muscular work ceases.

Many coenzymes are closely related to *vitamins* and are the derivatives of vitamins. The B group vitamins (except biotin and lipoic acid) function as parts of some coenzyme, *e.g.,* CoA is a derivative of *pantothenic acid.* When there is vitamin deficiency the coenzyme concentration decreases. Consequently enzyme function is depressed. Nucleotides may also function as coenzymes in certain metabolic reactions.

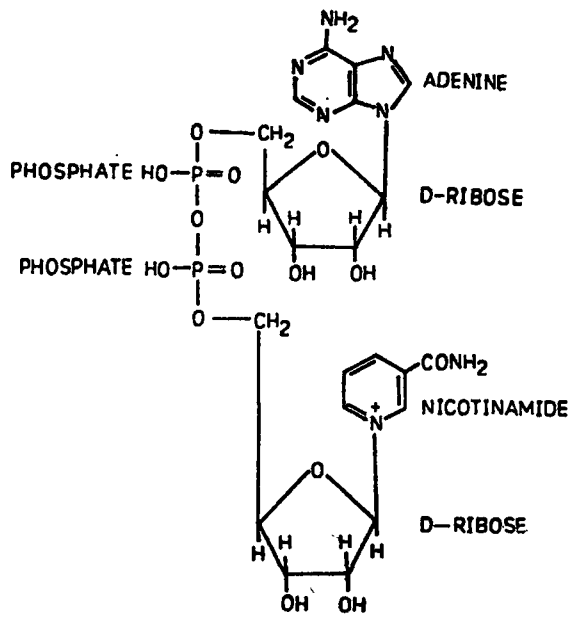
In some cases enzymes may be activated by simple substances like metal ions which are then called *activators.* The substrate forms a complex with the metal ion and then reacts with the enzyme. Examples of metal ions which function as cofactors are Na^+, K^+, Ca^{2+} Co^{2+}, Mg^{2+}, Cd^{2+}, Fe^{2+}, Cr^{3+} and Al^{3+}.

Structure of coenzymes : Metallic ions, vitamins and other types of organic compounds have been found to function as prosthetic groups or coezymes. An essential component of most of coenzymes is generally phosphate in the form of nucleotides. For example, nicotinamide ademine dinuleotides, also known as coenzyme I (NAD) or coenzyme II (NADP) act as coenzymes for dehydrogenases. Iron containing cytochromes make the electrons to get transferred from flavoproteins to cytochrome oxidase. Pyruvate decarboxylase is an enzyme that splits pyruvate into CO_2 and acetaldehyde in the yeast cells. It contains thiamine (vitamin B_1) as a part of its coenzyme.

Classification of coenzymes : The coenzymes have been classified in accordance to the reactions they participate in. Such a classification with their corresponding vitamins has been summarised in Table 9.3.

Mechanism of action of coenzymes : Now we will describe the chemical structure and mechanism of action of some important coenzymes.

1. Coenzyme I : It is also known as *cozymase,* DPN (diphosphopyridine phosphate nucleotide), codehydrogenase I or NAD^+ (nicotinamide adenine dinucleotide).



Coenzyme I (HAD^+)

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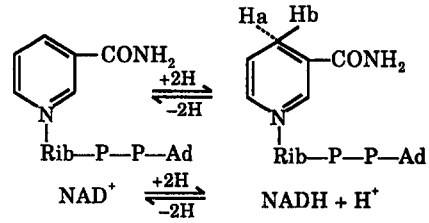
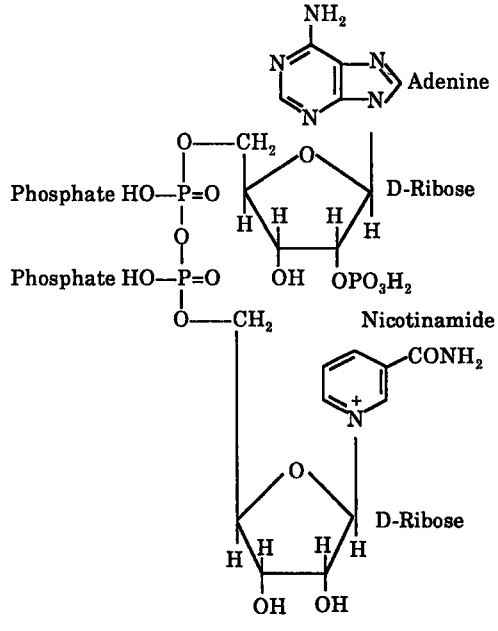
Coenzyme I is mainly involved in many hydrogen transferring reactions. In these reactions, H is added to a substrate from —C-atom of the pyridine nucleus. The pyridine gets reduced thereby retaining only two double bonds.



From the experimental work, it could be concluded that NAD^+ enzyme complex is usually stereospecific, only one hydrogen (H_a or H_b) is reacting exclusively. Which face of NAD^+ is attacked and which hydride ion from NADH is transferred depends upon the nature of the enzyme.

NADH absorbs light at 340 nm whereas NAD^+ shows no absorption at that wavelength. Thus, when NADH is formed during a reaction, absorption of light at 340 nm increases. The increase in absorption has been found to be proportional to the enzyme activity. This property finds use extensively in the bioassay of dehydrogenases and other oxidoreductases which employ NADH as coenzyme.

2. Coenzyme II : It is also known as *phosphocozymase,* TPN (triphosphopyridine nucleotide), *codehydrogenase II* or $NADP^+$ (nicotinamide adenine dinculeotide phosphate). This coenzyme has one additional phosphate group than coenzyme I molecule in position 2′ of ribose molecule of adenosine.



The role of coenzyme II is similar to that of coenzyme I.

3. Adenosine monophosphate, adenosine diphosphate and adenosine triphosphate : These coenzymes are involved in transphosphorylation. The structure of adenosine triphosphate is shown as follows :

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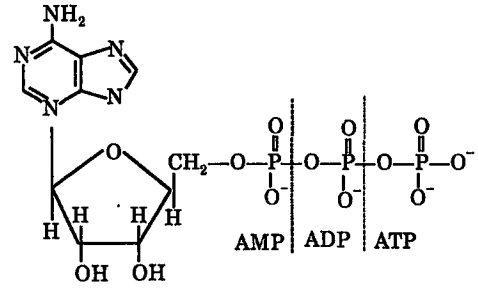


Adenosine triphosphate is a polyelectrolyte molecule having four negative charges. Since it has low molecular weight, it moves freely in cells. It acts as a mediator to receive energy from one reaction and transfers this energy to drive another reaction. ATP is synthesised in several metabolic reactions of the cell (cyclic, non-cyclic phosphorylation and oxidative phosphorylation).

In order to overcome the energy barriers, the energy must be supplied. In biosynthetic processes, ATP supplies this energy in transphosphorylation reactions in the presence of a suitable enzyme and it is converted into adenosine diphosphate (ADP).

$$ROH + ATP \longrightarrow R{-}OPO(OH)_2 + ADP$$

ADP also behaves as a phosphorylating agent and it is converted into adenosine monophosphate (AMP).

$$ROH + ADP \longrightarrow R{-}OPO(OH)_2 + AMP$$

A less usual reaction of ATP is pyrophosphorylation, *e.g.*,

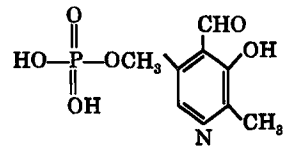
$$ROH + ATP \longrightarrow R{-}OPO(OH){-}OPO(OH)_2 + AMP$$

If the structural formulae of ATP, ADP and AMP (given above) are inspected, it can be seen that the phosphate bond in AMP is linked by the normal ester bond while the terminal phosphate groups in ADP and ATP are linked to a phosphate group by an acid anhydride bond. In hydrolytic reactions, the free energy change (heat of reaction) of an ester bond has been found to be ~ – 4.0 to – 12.5 kJ mol^{-1} while that for the acid anhydride bond is ~ – 33.5 kJ mol^{-1}. Therefore, there is a net free energy change of – 29.5 to – 21.0 kJ mol^{-1} in transphosphorylation reactions involving ADP and ATP. This free energy is used to drive coupled reactions. The acid anhydride bonds are known as energy-rich bonds. These are generally represented by the symbol. For example, ATP and ADP may be represented as follows :

ATP : Adenine—ribose—O—PO(OH)~O—PO(OH)~O—$PO(OH)_2$

ADP : Adenine—ribose—O—PO(OH)~O—$PO(OH)_2$

4. Pyridoxal phosphate (codecarboxylase) : It is involved in a number of important metabolic reactions of the α-amino acids, *e.g.*, transmination, racemisation, decarboxylation and elimination reactions.



Pyriodoxal Phosphate (PALP)

Role : Nearly all pyridoxal enzymes catalyse reactions of amino acids racemisation, decarboxylation, transamination, elimination of substituents and others. All the reactions can be described by the same mechanism.

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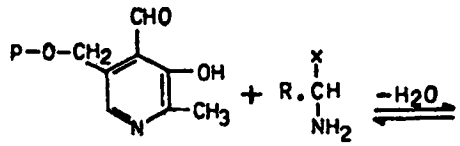
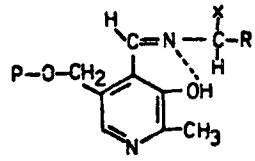
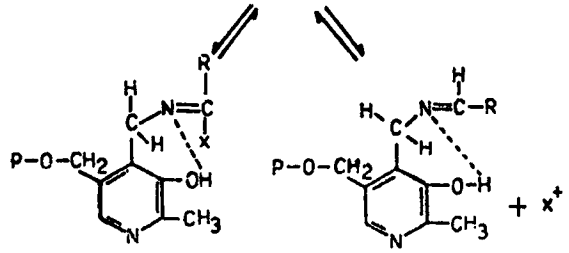
Pyridoxal phosphate is attached to the apoenzyme tightly; the aldehyde group of pyridoxal reacts with amino acids to form a Schiff's base. For racemisation, there occurs a shifting of electrons in Schiff's base, thereby leading to labilisation of all bonds around the α-carbon atom of the amino acid.

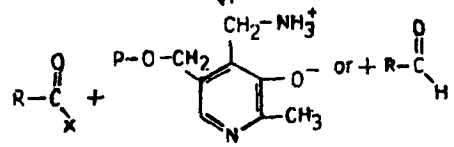




SCHIFFS BASE



DECARBOXYLATION
OCCURS IF × 15 COOH

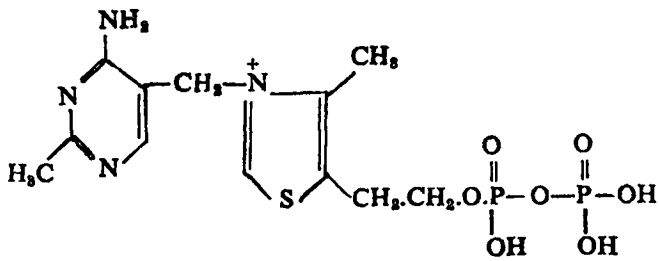


PYRIDOXAMINE PHOSPHATE

Pyridoxal phosphate also acts as a coenzyme reaction between amino acids and keto acids.

This reaction, of great importance biologically, is catalysed by pyridoxal phosphate and its mechanism is similar to racemisation.

5. Thiamine pyrophosphate (cocarboxylase): It is involved in the transfer of acyl and carboxyl groups. It is derived from the vitamin, thiamine (vitamin B_1).



Cocarboxylase

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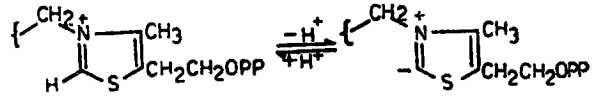
Action : The enzyme *carboxylase* breaks down pyruvic acid into acetaldehyde in fermentation and carbohydrate metabolism. This reaction takes place in the presence of coenzyme, *cocarboxylase.*

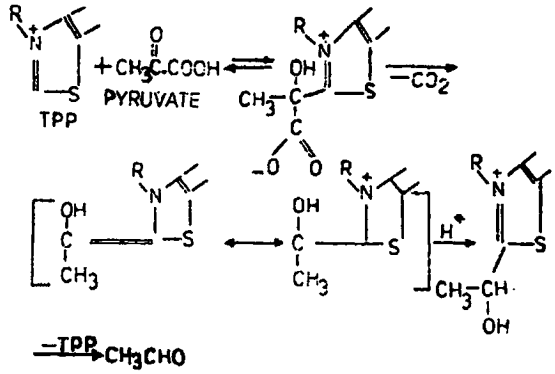
$$CH_3COCOOH \xrightarrow[\text{Cocarboxylase}]{\text{Carboxylase}} CH_3CHO + CO_2$$

Pyruvic acid

The mechanism of the cocarboxylase depends upon the ionisation of the proton at C—2 in the thiazolium ring. The liability of this hydrogen atom is by its ready displacement by deuterium when thiazolium salts are dissolved in acidified deuterium oxide.

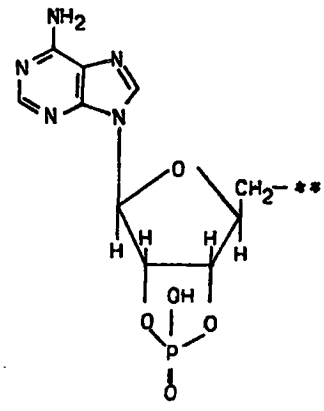


The most important reaction catalysed by TPP is the decarboxylation of pyruvic acid to acetaldehyde. The reaction mechanism is given as follows :



The decarboxylation of pyruvic acid by this mechanism involves a non-enzymatic process and in it there occurs transfer of electron pairs. In all other thiamine catalysed enzymic reactions also, the mechanism has been found to be almost similar. Thiamine cleaves the carbonyl —x bond, where x is generally—H or —COO^- group. A substrate-thiamine complex is later on cleaved at α-carbon atom to liberate the coenzyme.

6. Coenzyme A : This coenzyme is a complex thiol derivative and is usually written as CoA—SH but CoA is also in common usage. In contrast to coenzyme I and coenzyme II, it is not an oxidising-reducing coenzyme but it is acylating; it is an acyl transfer coenzyme and a cofactor for a wide variety of biologically acylations, *e.g.,* the formation of acetoacetate from acetate in pigeon liver.



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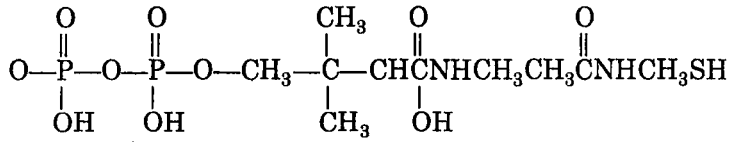
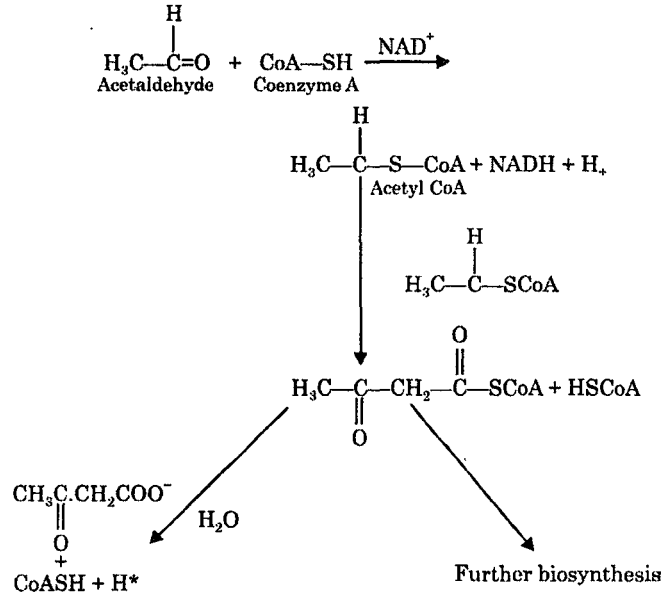
where denotes the following groups :**



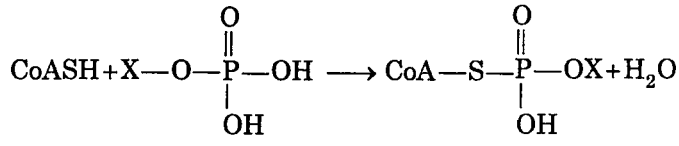
Role : Coenzyme A is required for the acetylation reactions. Coenzyme A accepts acetyl groups from one metabolite and donates them to another in the presence of specific enzymes.

The most important CoA compound is acetyl CoA (activated acetate), which takes part in many acyl transferring reactions. In this, the acyl residue is bound to free —SH groups to form very reactive thioester.

In the formation of aceto acetyl CoA from acetate thioester there occurs nucleophilic displacement at the carbon and one more acetate group gets added at the place of coenzyme A.



Besides, acylation, coenzyme A can also undergo phosphorylation.



In the reactions catalysed by phosphotransacetylase, thiokinase and phosphokinase, phosphorylation of CoA occurs. Thiokinases have been found to catalyse the following reaction by involving CoA.

acetyl CoA + adenylic acid + $2H_3PO_4$ ⟶ ATP + acetate + CoA—SH

Coenzyme A also takes part in the elimination reaction. For example, in the formation of unsaturated fatty acids from saturated fatty acid coenzyme A is involved. This needs another coenzyme FAD.

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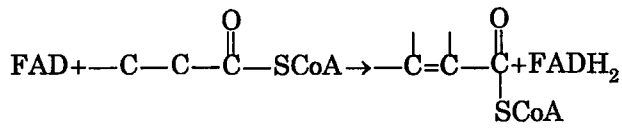


Flavin nucleotides : There are two types of flavin nucleotides that take part in the respiratory chain; flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FMN consists of phosphate, ribitol, and flavin bonded together as shown in Fig. 9.17 (*a*). The combination of ribitol and flavin forms riboflavin (vitamin B_2). FAD is the combination of FMN and AMP jointed by an anhydride inkage [Fig. 9.17 (*b*)].

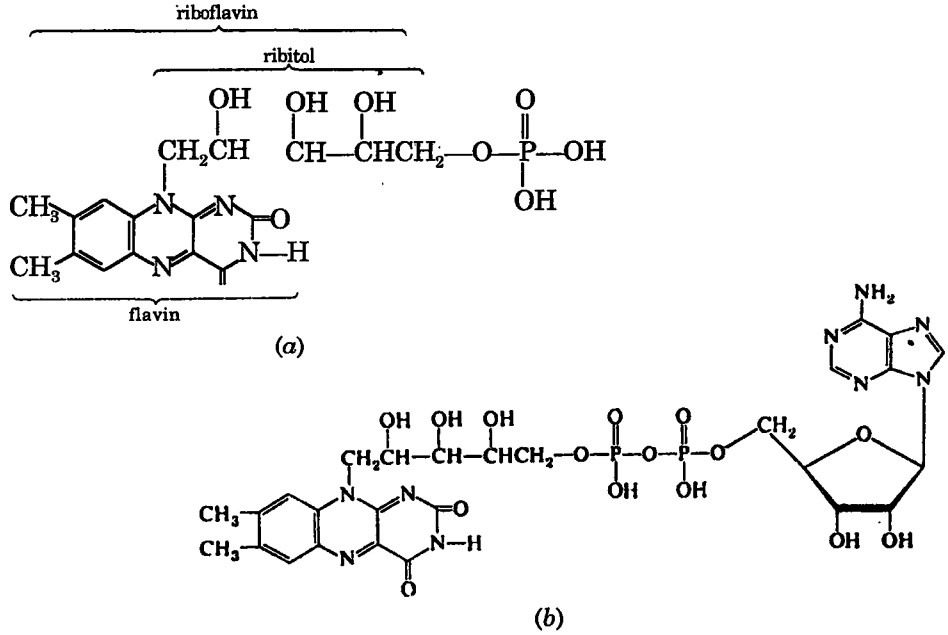


Fig. 9.17. Flavin nucleotides. (*a*) Flavin mononucleotide, FMN.
(*b*) Flavin adenine dinucleotide, FAD.

Hydrogens are picked up and released by the flavin portion of the molecule to form reduced and oxidized forms of the coenzymes :

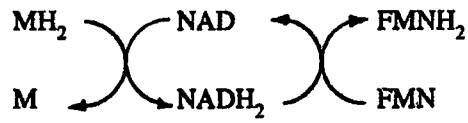
Oxidation of $FMNNH_2$: $FMNH_2 \longrightarrow FMN + 2H$

Reduction of FMN : $FMN + 2H \longrightarrow FMNH_2$

Oxidation of FADH : $FADH_2 \longrightarrow FAD + 2H$

Reduction of FAD : $FAD + 2H \longrightarrow FADH_2$

In the electron transport system, FMN removes hydrogens from $NADH_2$. This makes NAD available to oxidize additional metabolities.



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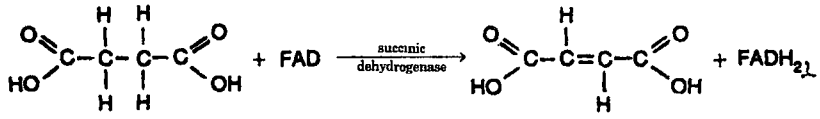
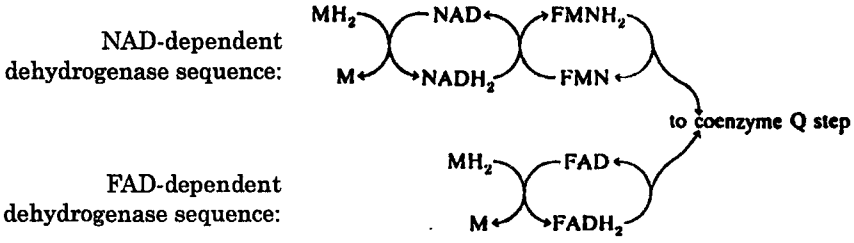
A metabolite may react directly with FAD. The coenzyme is used primarily during reactions in which hydrogens are removed from adjacent carbon atoms during conversion of carbon-carbon single bonds to double bonds.

During carbohydrate and lipid metabolism, the conversion of succinic acid to fumaric acid involves such a change. The reaction is catalyzed by succinic dehydrogenase through the action of its coenzyme, FAD. In this case, enzyme action directs the formation of the trans isomer exclusively.



Succinic Acid Fumaric Acid

Both FMN and FAD are coenzymes for dehydrogenases. If the metabolite is FAD dependent, the metabolite loses hydrogens to FAD directly. A metabolite which is NAD dependent initially loses hydrogens to NAD, which passes them on to FMN.



In either case, the reduced flavin nucleotide interacts with coenzyme Q, the next carrier in the respiratory chain.

8. Coenzyme Q : It is a lipid material whose role has only recently been completely established. The coenzyme can exist in oxidized and reduced forms, a role consistent with its activity in an oxidation-reduction sequence.

Reduction : $CoQ + 2H \longrightarrow CoQH_2$ (reduced form)

Oxidation : $CoQH_2 \longrightarrow 2H + CoQ$ (oxidized form)

CoQ accepts hydrogens from $FADH_2$ or $FMNH_2$, causing oxidation of the $FADH_2$ or $FMNH_2$ and reduction of CoQ.

It has been experimentally demonstrated that sodium amytal, a barbiturate, blocks electron transfer from $FMNH_2$ to coenzyme Q.

9.9 Isoenzymes

Isoenzymes or isozymes are families of oligomeric enzymes which catalyse the same reaction but differences in their subunit composition modify the rate at which each molecular species transforms substrate. Isoenzymes may be divided into primary or secondary isoenzymes. Primary isoenzymes are the products of multiple gene loci which code for distinct protein molecules or are the products of multiple alleles at a single gene locus (called alloenzymes). Secondary isoenzymes are derived by post-translational modifications including glycosylation. Because of differences in

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their amino acid composition, primary isoenzymes may be detected by variations in their electrophoretic mobility.

More than a hundred enzymes are known to exist as isozymes. When the variants of an enzyme are within the same species of an organism they are called *intra-specific or ontogenetic variants.* When they are from different species they are called interspecific or *phylogenetic variants.*

Multiple forms of the enzyme may also be found in the same organism. In humans there are three different types of *hemoglobin,* two in the adult and one in the foetus. These are formed by the union of four polypeptide chains. The enzyme *lactic dehydrogenase* (LDH) is present in most animal tissues as five isozymes. It has two subunits or monomers, the H (heart) polypeptide and the M (muscle) polypeptide. Heart and muscle LDH have different amino acid compositions and different properties. Four units, in different combinations make up the five different molecular forms of LDH. The five isozymes are.

1. HHHH or H_4
2. HHHM or H_3M
3. HHMM or H_2M_2
4. HMMM or HM_3
5. MMMM or M_4

Fig. 9.18 Molecular forms of LDH.

The M subunit is formed under conditions of low oxygen tension, and the H subunit under conditions of plentiful oxygen supply (aerobic metabolism). It has been found that embryos metabolize through low oxygen supply mechanisms, and in the course of development the metabolism becomes more oxygen dependent. As development proceeds the M_4 and HM_3 forms of LDH diminish, and are replaced by the H_4 and H_3M forms.

The tissue distribution of LDH isoenzymes, however, demonstrates major variation from species contributing to the difficulties in the provision of a rational explanation of the physiological roles of A and B homo and heterotetramers. Rather than variations in enzyme kinetics, other biological advantages such as differential binding to subcellular organelles or differential rates of protein degradation may be important.

Within any vertebrate including man most organs demonstrate different LDH isoenzyme patterns. Being a cytoplasmic isoenzyme, LDH_1 increases significantly in the blood circulation after a heart attack because of its release from damaged cells. This is one example of the use of isoenzyme levels in the diagnosis of clinical disorders.

Isozymes may be *homologous or analogous. Homologous* isozymes have essential similarly molecular structure and catalytic properties. Cytochrome *c* from different organisms shows homology. Half the amino acids of cytochrome *c* of such widely separated organisms like yeasts and mammals are identical. Generally isozymes of more closely related organisms show greater similarity than isozymes of widely separated organisms. For example, cytochrome c from humans and moths have 75 common residues, while those from humans and yeasts have 65 common residues. *Analogous* isozymes have similar reaction but different molecular structure and catalytic properties. They have arisen from different ancestral genes.

Isozymes provide a clue to the genetic relationships of organisms. The sequence of amino acids in an enzyme is related to the structure of DNA. Therefore, similarity in isozymes is correlated with similarity in DNA structure.

9.10 Allostery

Some enzymes when their v_0 is plotted as a function of [S] do not show the hyperbolic curve but a sigmoidal curve (Fig. 9.19). The rate of the reaction at a given [S] is increased or decreased

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by the addition of specific substances, *i.e.*, activators or inhibitors. Enzymes which exhibit this behaviour are called allosteric enzymes based *on a concept developed earlier to explain the oxygenation*/deoxygenation of hemoglobin.

In addition to the substrate-binding site (active site), allosteric enzymes possess other sites to which inhibitors and activators, termed allosteric effectors, may bind and influence catalytic events through induced conformational changes in the enzyme (Figure 9.20).

Most allosteric enzymes contain numerous subunits. In most of these enzymes., *e.g.*, pyruvate carboxylase, both catalytic and regulatory sites are located on the same polypeptide chain whilst some, *e.g.*, aspartate carbamoyl transferase, carry the catalytic and regulatory sites on different subunits. Through the sigmoidal shape of their v_0 against [S] plot [Figure 9.19], X-ray crystallographic and other studies, it is known that many allosteric enzymes display cooperativity of substrate binding even in the absence of allosteric effectors. This cooperativity may be positive or negative. In positive cooperativity, when one binding site is occupied, subsequent binding to other sites occurs more readily. In negative cooperativity, successive binding occurs with decreasing ease. In the presence of an allosteric activator, the curve tends towards a hyperbola, whilst in the presence of an inhibitor, the curve becomes more sigmoidial. The structure of the allosteric enzyme is particularly suited to a role in the regulation of metabolic pathways. The end products of a metabolic pathway frequently bind to an allosteric site on the first enzyme of the pathway as a negative effect or to reduce the rate of synthesis of the end product. This form of control is called feedback inhibition (Figure 9.21). As the end product F increases in concentration, it diffuses to the allosteric enzyme (E_A) causing a reduced synthesis of its product B which, in turn, lowers enzymic reaction rates in the remainder of the pathway. Conversely, allosteric enzymes may be stimulated by positive effectors which are not end products of pathways but other metabolites that induce conformational changes in enzymes to enhance their activities. These allosteric enzymes are termed heterotropic since the effector and substrate are different ligands. In some enzymes, the substrate creates a positive cooperative effect by binding to the catalytic sites not allosteric sites. Such enzymes are called homotropic. This homotropic cooperativity is advantageous in the utilization of accumulated substrate and may also be useful because it enables larger rate changes for smaller changes in the substrate concentration.

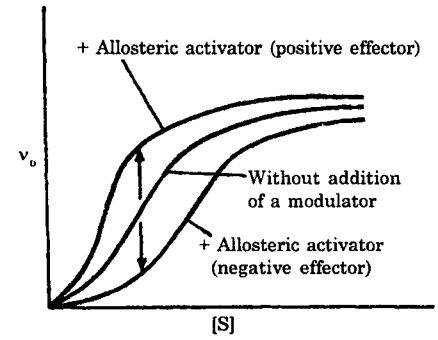

Fig. 9.19 (Effect of substrate concentration on the initial velocity of an allosteric enzyme in the presence and absence of specific modulators.

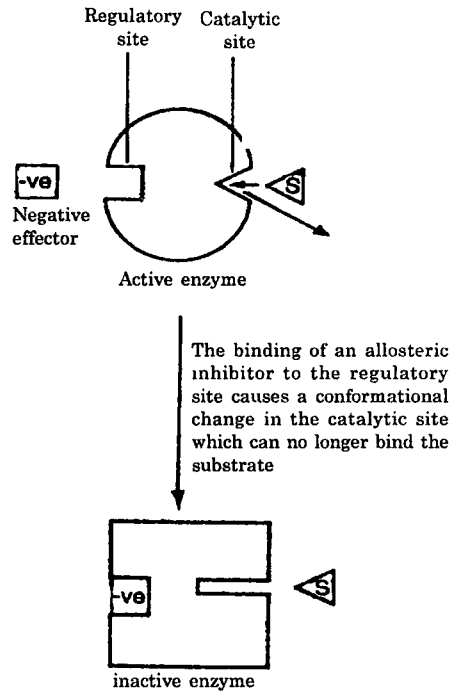

Fig. 9.20 Induced conformational change in an allosteric enzyme.

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Two main models, the concerted (or symmetry) model of Monod, Wyman and Changeux (MWC) and the sequential model of Koshland, Nemethy and Filmer (KNF), form the basis of approaches to explain the sigmoidal relationship between v_0 and [S] (Figure 9.21) in molecular terms. Although most allosteric enzymes are composed of at least four subunits, for simplicity the principles of these models will be outlined employing a dimer.

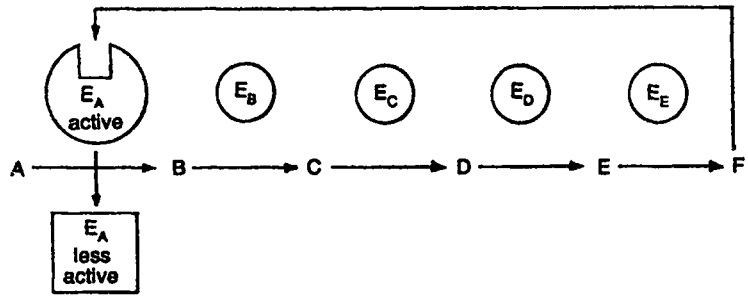

Fig. 9.21. Feedback inhibition.

(*i*) The MWC model proposes that two different conformation states, called R and T, exist in equilibrium (Figure 9.22). The R (relaxed) conformation is composed of subunits which have a high affinity for substrate and activator and is therefore the active from. The T (tense) conformation is composed for subunits which have a high affinity for inhibitor but low affinity for substrate and is the inactive form. The basis of this model is that both subunits of the dimer can only exist in the same conformational state, *i.e.*, RR or TT but not RT conformations. The RR and TT conformations exist in equilibrium. In the absence of activator, the TT form predominates. When the first activator molecules bind to the few RR molecules, the equilibrium responds by a conformational shift from TT to RR to produce more active enzyme molecules which increase the rate of substrate transformation. Progressive increases in activator concentration result in a concomitant increase in TT to RR conversions. The sigmoid curve then approaches the non-allosteric hyperbolic plot. Inhibitors may bind to the TT conformation, the equilibrium responds by means of RR to TT conversions with a concomitant reduction in active sites. The sigmoid curve then becomes more pronounced. Negative cooperativity cannot be accounted for by this model.

(*ii*) The KNF model is an extension of the principle of induced fit. The effectors modify subunit conformation directly and not through a shift in an assumed equilibrium between two forms. This model proposed that ligand binding sites within analogous conformations may have differing affinities for the same ligand. The binding of an effector to its allosteric site in one subunit induces conformational changes within that subunit which then

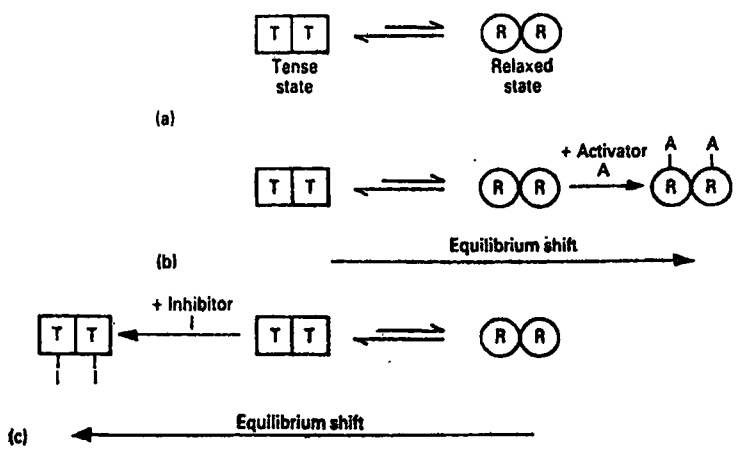

Fig. 9.22. The concerted (or symmetry) model of Monod, Wyman and Changeux (MWC) (*a*) Initial equilibrium. (*b*) In the presence of activator. (*c*) In the presence of inhibitor.

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induces conformational changes in the other subunit. A conformation equivalent to RT which is not allowed in the MWC model is therefore a requisite of the KNF model. If the effector is an activator [Figure 6.23 (*a*)], its binding to the first subunit will effect a conformational change that will enhance substrate binding to the same subunit and also induce a conformational change in the second subunit to increase its affinity firstly for the activator and then for substrate. Inhibitors function in lowering the affinity of the active sites for the substrate through conformational changes in both subunits [Figure 9.23 (*b*)].

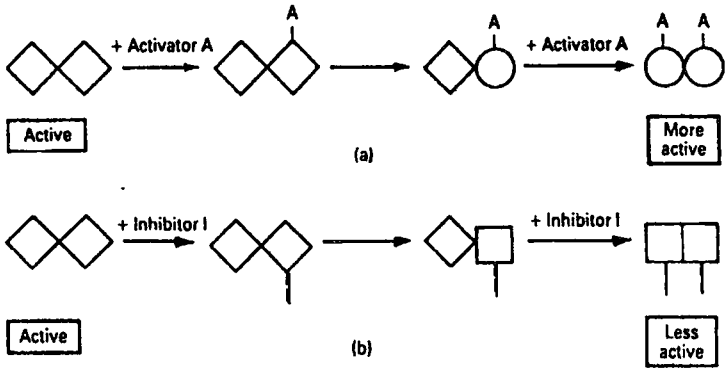

Fig. 9.23. The sequential model of Koshland, Nemethy and Filmer (KNF). Allosteric effect of (*a*) an activator and (*b*) an inhibitor.

Negative cooperativity may be accounted for by this model. Neither model can satisfactorily explain the action of some allosteric proteins for which more complex models are required.

9.11 Enzymes in Medical Diagnosis and Treatment

Most enzymes are confined within the cells of the body. However, small amounts of enzymes can also be found in body fluids such as blood, urine, and cerebrospinal fluid. The level of enzyme activity in these fluids can be easily monitored. It has been found that abnormal activity (either high or low) of particular enzymes in various body fluids signals either the onset of certain diseases or their progress. Table 9.4 lists some enzymes used in medical diagnosis and their activities in normal body fluids.

A number of enzymes are assayed during myocardial infarction in order to diagnose the severity of the heart attack. Dead heart muscle cells spill their enzyme contents into the serum. Thus, the level of glutamate-oxaloacetate transaminase (GOT) in the serum rises rapidly after a heart attack. Together with GOT, lactate dehydrogenase (LDH) and creatine phosphokinase (CK) levels are monitored. In infectious hepatitis, the glutamate-pyruvate trasaminase (GPT) level in the serum can rise to ten times normal. There is also a concurrent increase in GOT activity in the serum.

Enzyme Units

Enzyme activity may be expressed in a number of ways. The commonest is by the initial rate (V_0) of the reaction being catalyzed (*e.g.*, μmol of substrate transformed per minute; μmol min^{-1}). There are also two standard units of enzyme activity, the *enzyme unit* (*U*) and the *katal* (*kat*). An enzyme unit is that amount of enzyme which will catalyze the transformation of 1 μmol of substrate per minute at 25°C under optimal conditions for that enzyme. The katal is the accepted SI unit of enzyme activity and is defined as that catalytic activity which will raise the rate of a reaction by one mole per second in a specified system. It is possible to convert between these different units of activity using 1 μmol min^{-1} = 1 U = 16.67 nanokat. The term *activity* (or

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total activity) refers to the total units of enzyme in the sample, whereas the *specific activity* is the number of enzyme units per milligram of protein (units mg^{-1}). The specific activity is a measure of the purity of an enzyme; during the purification of the enzyme its specific activity increases and becomes maximal and constant when the enzyme is pure.

Enzyme Assays

The amount of enzyme protein present can be determined (assayed) in terms of the catalytic effect it produces, that is the conversion of substrate to product. In order to *assay* (monitor the activity of) an enzyme, the overall equation of the reaction being catalyzed must be known, and an analytical procedure must be available for determining either the disappearance of substrate or the appearance of product. In addition, one must take into account whether the enzyme requires any *confactors*, and the *pH* and *temperature* at which the enzyme is optimally active. For mammalian enzymes, this is usually in the range 25–37°C. Finally, it is essential that the rate of the reaction being assayed is a measure of the enzyme activity present and is not limited by an insufficient supply of substrate. Therefore, very high substrate concentrations are generally required so that the *initial reaction rate*, which is determined experimentally, is proportional to the enzyme concentration.

An enzyme is most conveniently assayed by measuring the rate of *appearance of product* or the the *rate of disappearance of substrate*. If the substrate (or product) absorbs light at a specific wavelength, then changes in the concentration of these molecules can be measured by following the *change of absorbance* at this wavelength. Typically this is carried out using a *spectrophotometer*. Since absorbance is proportional to concentration, the rate of change in absorbance is proportional to the rate of enzyme activity in moles of substrate used (or product formed) per unit time.

Two of the most common molecules used for absorbance measurement in enzyme assays are the coenzymes *reduced nicotinamide adenine dinucleotide* (*NADH*) and *reduced nicotinamide adenine dinucleotide phosphate* (*NADPH*) which each absorb in the ultraviolet. (UV) region at 340 nm. Thus, if NADH or NADPH is produced during the course of the reaction there will be a corresponding increase in absorbance at 340 nm, whilst if the reaction involves the oxidation of NADH or NADPH to NAD^+ or $NADP^+$ respectively, there will be a corresponding decrease in absorbance, since these oxidized forms do not absorb at 340 nm. One examples, is that the activity of *lactate dehydrogenase* with lactate as substrate can be assayed by following the increase in absorbance at 340 nm, according to the following equation :

$$\underset{\text{lactate}}{CH_3CH(OH)COO^-} + NAD^+ \rightleftharpoons \underset{\text{pyruvate}}{CH_3COCOO^-} + NADH + H^+$$

Linked Enzyme Assays

Numerous reactions do not involve substrates or products that absorb light at a suitable wavelength. In this case it is often possible to assay the enzyme that catalyzes this reaction by *linking* (or *coupling*) it to a second enzyme reaction that does involve a characteristic absorbance change. For example, the action of the enzyme glucose *oxidase*, which is often used to measure the concentration of glucose in the blood of diabetic patients, does not result in a change in absorbance upon conversion of substrates to products (Fig. 9.24). However, the hydrogen peroxide produced in this reaction can be acted on by a second enzyme, peroxidase, which simultaneously converts a colourless compound into a coloured one (*chromogen*) whose absorbance can be easily measured (Fig. 9.24). If the activity of the first enzyme (glucose oxidase) is to be measured accurately, the second enzyme (peroxidase) and its co-substrates or coenzymes must be in excess

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so as not to be the *rate-limiting step* of the linked assay. This will ensure that the rate of production of the coloured chromogen is proportional to the rate of production of H_2O_2 whose production in turn is proportional to the activity of glucose oxidase.

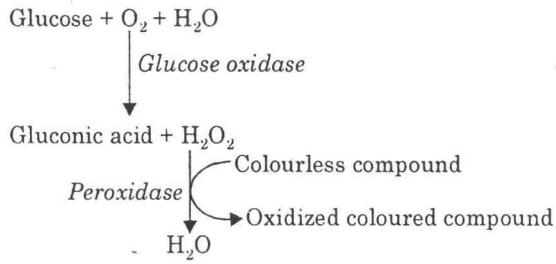

Fig. 9.24. A linked enzyme assay with glucose oxidase and peroxidase can be used to measure the amount of glucose in a blood sample.

Table 9.4

ENZYME ASSAYS USEFUL IN MEDICAL DIAGNOSIS

Enzyme	*Normal Activity*	*Body Fluid*	*Disease of Diagnosis*
Acid phosphatase	2.5-12 mlU/mL[a]	Serum	Prostate cancer
Alkaline phosphatase	13.-38 mlU/mL	Serum	Liver or bone disease
Amylase	40-160 SU/100mL	Serum	
	50000-80000 SU/100Ml	Pancreatic fluid	Pancreatic disease or mumps
Glutamateoxaloacetate transaminase (GOT)	7-19 mlU/mL	Serum	
	7-49 mlU/mL	Cerebrospinal fluid	Heart attack or hepatitis
Glutamate-pyruvate transaminase (GPT)	3-17 mlU/mL	Serum	Hepatitis
Lactate dehydrogenase (LDH)	150-450 WU/mL	Serum	
Creatine Phosphokinase (CK)	7-60 mlU/mL	Serum	Heart attack
Phosphohexoseisomerase (PHl)	20-90 mlU/L	Serum	

(*a*) mlU = millinternational units; U = Somogyi units; WU = Wrobleski units.

In some cases, the administration of an enzyme is part of therapy. After duodenal or stomach ulcer operations, patients are advised to take tablets containing digestive enzymes that are in short supply in the stomach after surgery.

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10 Chemistry of Alkaloids

10.1 Introduction

It is not easy to give an exact definition of what is meant by the term *"alkaloids."* However, the term "*alkaloids*" was first of all introduced by W. Meissner in 1819. According to him, "*Alkaloids* (which mean alkali-like, *alk alkaloid like*) *were defined as basic-nitrogen compounds isolated from plants.*"

The above definition of alkaloids which covers an extraordinary wide variety of compounds underwent many changes as more of the alkaloids were isolated and studied. In order to incorporate these changes, Konigs (1880) suggested that the definition of alkaloids should be as follows :

"*Alkaloids should be defined as naturally occurring organic bases which contain a pyridine ring.*"

Again, the above definition is not complete because it embraces only a limited number of compounds and, again, the definition was modified by Ladenburg. According to him, "*Alkaloids are defined as natural plant compounds that have a basic character and contain at least one nitrogen atom in a heterocyclic ring.*"

Again, the above definition is not complete because it excludes many synthetic compounds and many compounds obtained from animal sources.

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With the discovery of more alkaloids, two more characteristics were added to the above definition of alkaloids. These characteristics are :

(a) Complex molecular structure, and

(b) Significant pharmacological activity.

By keeping the above points in view, the alkaloids were defined as "*basic nitrogeneous plant products, mostly optically active and possessing nitrogen heterocyclic as their structural units, with a pronounced physiological action.*"

The above definition of alkaloids must be interpreted with some care because some compounds although alkaloids do not confine to the definition, while other compounds which are not alkaloids confine to this definition.

10.2 Occurrence of Alkaloids

Alkaloids are a chemically heterogeneous group of approximately 2,500 basic nitrogen containing substances found in about 15 per cent of all vascular land plants and in more than 150 plant families. They are widely distributed in higher plants particularly the dicotyledons (in abundance in the families *Apocynaceae, Papaveraceae, Papilionaceae, Ranunculaceae, Rubiaceae, Rutaceae and Solanaceae*) but less frequently in lower plants and fungi.

In plans, alkaloids, due to their basic nature, generally exist as salts of organic acids like acetic, oxalic, citric, malic, lactice, tartaric, tannic, aconitic acids, etc. Some feeble basic alkaloids like nerceine, nicotine, etc., occur free in nature. A few alkaloids also occur as glycosides of sugars like glucose, rhamnose and galactose (*e.g.,* alkaloids of *Solanum and Veratrum groups*), as amides (piperine) or as esters (atropine, cocaine), of organic acids. Also, the structurally related alkaloids generally occur in the form of salts of the same acid, *e.g.,* cinchona alkaloids with quininic acid, aconite alkaloids with aconite acid, and opium alkaloids with neconic acid.

10.3 Classification of Alkaloids

There are several methods of classification. Some of these are as follows :

(a) Taxonomic : This is done according to family. Thus, alkaloids may be described as *Solanaceous or Papilionaceous* without reference to the chemical type of alkaloid present. Since both families contain alkaloids of several types (solanaceae-tropane, pyridine, steroidal; papilionaceae quinolizidine and pyrrolizidine), then the disadvantages of the systems are obvious. It is more usual to describe alkaloids according to the genus in which they occur, *e.g., Ephedra, Cinchona. etc.*

(b) Pharmacological : Pharmacological classification lists alkaloids according to their use or physiological activity, *e.g.,* analgesic alkaloids, cardioactive alkaloids, etc. Whereas alkaloids within a group frequently have chemical similarities, this is by no means the rule.

(c) Chemical : The chemical classification of alkaloids is universally adopted and depends on the fundamental (frequently heterocylic) ring structure present. Thus, quinine is regarded as a quinoline type, papaverine and isoquinoline and ergometrine an indole. On the other hand, morphine, which is normally regarded as a phenanthrene derivative, could easily be included with the quinolines; thus to some extent chemical classification depends on the convention adopted.

On the basis of chemical classification, numerous classes of alkaloids are possible. Following are names of such classes and some examples of classes :

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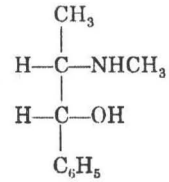
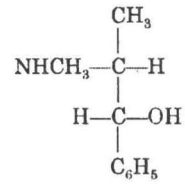
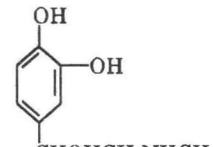
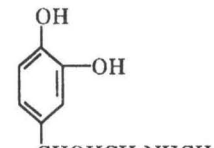
1. *Phenylethyl amino alkaloids.* Examples are as follows :

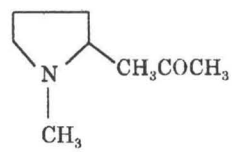

D(–)-Ephedrine

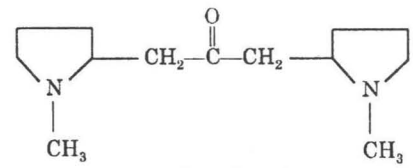

L(+)-Ephedrine


Adrenaline

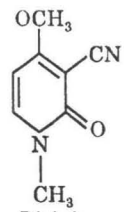

(±)-Noradrenaline

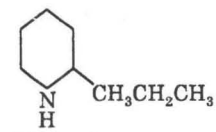
2. *Pyrrollidine Alkaloids.* Examples are as follows :

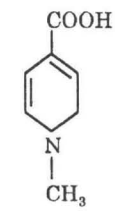

Hygrine

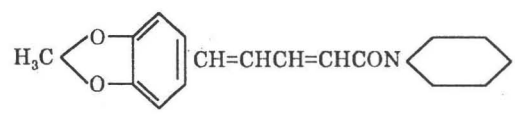

Cuscohygrine

3. *Pyridine or Piperine alkaloids.* Examples are as follows :

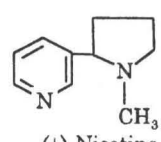

Ricinine

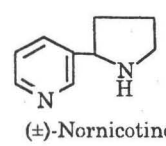

(±)-Coniine


Trigonelline


Piperine

4. *Pyrrolidine-pyridine group.* Examples are as follows :


(±)-Nicotine


(±)-Nornicotine

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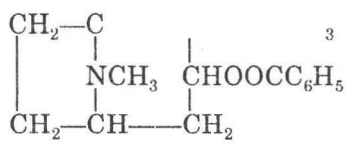
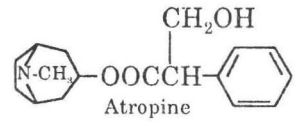
5. *Tropane alkaloids.* Some examples are as follows :

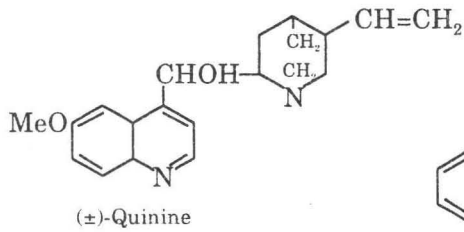


(–)-Cocaine

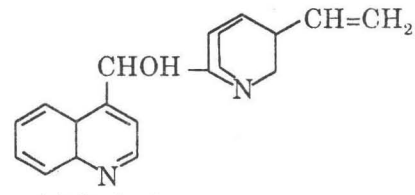


Atropine

6. *Quinoline alkaloids.* Some examples are as follows :

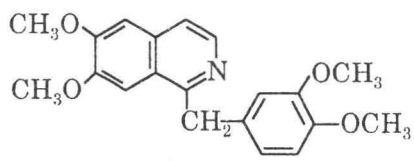


(±)-Quinine

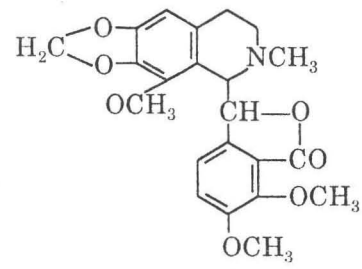


(±)-Cinchonine

7. *Isoquinoline alkaloids.* Some examples are as follows :

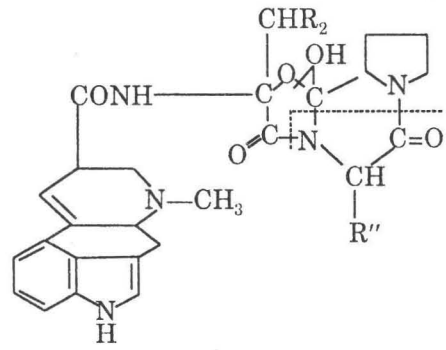


Papaverine



Narcotine

8. *Indole alkaloids.* Some examples are as follows :



In the above structure,

(*i*) When R = H, R″ = —$CH_2C_6H_5$, it is ergotamine.

(*ii*) When R = H, R″ = —$CH_2CH(CH_3)_2$, it is ergosine.

(*iii*) When R = — CH_3, R″ = —$CH_2C_6H_5$, it is ergocristine.

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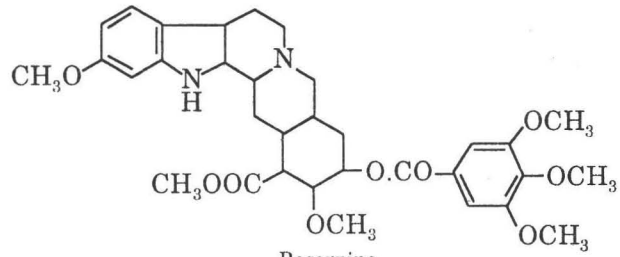
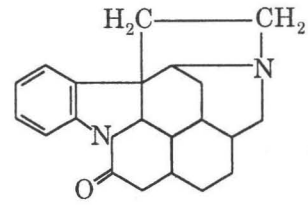
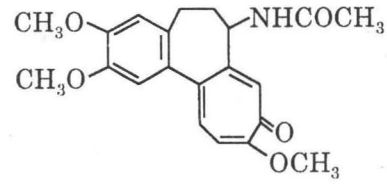


Reserpine



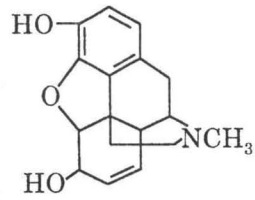
Strychnine

9. *Tropolone alkaloids.* An example is as follows :

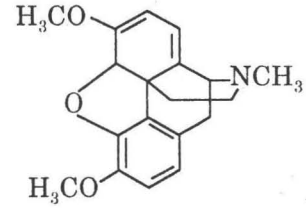


Colchicine

10. *Phenanthrene group.* Some examples are as follows :



Morphine



Thebaine

10.4 General Properties of Alkaloids

Some of the general properties of alkaloids are as follows :

(a) Most of the alkaloids are generally *colourless crystalline solids which are insoluble in water but soluble in organic solvents* like chloroform, alcohol, ether, etc. However, some alkaloids (*e.g.,* conine and nicotine) are liquids which are *soluble in water*. Also, a few alkaloids are coloured, *e.g.,* berberine is yellow.

(b) They are generally *bitter* in taste and are optically active; the majority being laevorotatory. The optically active alkaloids are often useful for resolving racemic acids.

(c) Nearly, all alkaloids due to their basic character, form crystalline salts with inorganic as well as organic acids. These salts, unlike the parent alkaloids are, generally soluble in water but insoluble in organic solvents. Parent alkaloids are obtained from these salts by treating them with bases. With chlorides of gold, platinum and mercury, they form double salts.

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(d) Alkaloids also yield insoluble precipitates when reacted with solution of phosphotungstic acid, phosphomolybdic acid, picric acid, potassium mercuri-iodide, etc. Many of these precipitates have definite crystalline shapes and so may be used for the identification of an alkaloid. Some of these reagents are being employed for detecting the alkaloids especially in paper and thin layer chromatography.

(e) Most of the alkaloids contain oxygen.

(f) Most of the alkaloids contain one or two nitrogen atoms usually in the tertiary state in a ring system. Most alkaloids react with methyl iodide to form crystalline adducts, the precise nature of the adducts depends upon whether the alkaloid is a secondary or tertiary base.

10.5 Biological Functions of Alkaloids

The function of alkaloids within the plants is not clearly understood but it is clear that they are not produced in plants for a single function but for many functions that are summarised as follows :

(*i*) They may act as reserve substances to supply nitrogen. However, there is little evidence that they are utilised in the conditions of nitrogen deficiency.

(*ii*) They may be the end-products of detoxification mechanisms otherwise their accumulation in plants may otherwise cause damage to the plants.

(*iii*) They may act as poisonous substances which afford plants safety from herbivores and insects.

(*iv*) They may function as plants stimulants or regulators similar to the hormones in the activities like growth, metabolism and reproduction.

(*v*) They may act as reservoirs for protein synthesis.

(*vi*) They are excretory products of plants and excess of ammonia is excreted by this method.

(*vii*) Inhibition of enzyme activity by alkaloids is also known.

It is interesting to note that 85 to 95 per cent plants carry out all their normal activities without involving alkaloids, thus indicating that the function of the alkaloids within the plants, if any, is still not understood clearly.

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11 Chemistry of Nucleic Acids

11.1 Introduction to Nucleic Acids

Nucleic acids, like protein, are macromolecules ranging in molecular weight from about 30,000 to several millions. The repeating unit in nucleic acid is the nucleotide whereas in proteins, the repeating units is α-amino acid. Nucleic acids derive their names because of their primary occurrence in the nucleus.

11.2 Chemical Composition of Nucleic Acids

Elements constituting nucleic acids are C, H, O, N and P. Most of the nucleic acids have approximately 15 to 16% nitrogen and 9 to 12% phosphorus.

Nucleic acids are colourless complex compounds which are made up of three units : bases (purine or pyramidine), sugar and phosphoric acid. These are obtained by the careful hydrolysis of nucleoproteins.

11.3 Classification of Nucleic Acids

Two types of nucleic acids have been known for a long time : *deoxyribonucleic acid* (DNA) and *ribonucleic acid* (RNA). DNA is found predominantely in the nucleus while RNA is predominant in the cytoplasm. The genetic information contained in the DNA molecule is essentially a set of coded instructions for the synthesis of proteins by living cells. DNA also synthesizes RNA, and RNA in turn is responsible for the synthesis of proteins. In this way, the nucleic acids determine the ultimate form and function of all living organisms.

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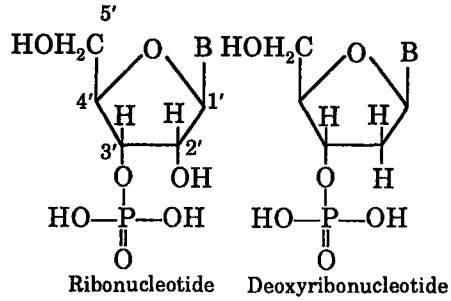
11.4 Relation Among Nucleic Acids, Nucleotides and Nucleosides

Nucleic acids : These are defined as polynucleotides (many nucleotides) in which repeating units are ribonucleotides (in RNA) or deoxyribonucleotides (in DNA). The difference between the two structures is that in deoxyribonucleotides there is no—OH group in position 2′. The presence of three esterifiable—OH groups in the ribose portion or two in the deoxyribose portion gives rise to several possible phosphoesters. The structures shown below are the 3′-phosphoesters.



Ribonucleotide Deoxyribonucleotide

Nucleotides are building blocks of large molecules. They serve three crucial functions in cells. Some are energy carriers, others are *co-enzymes* and still others are carriers of *hereditary information* (genetic code).

Nucleotides : Structurally, a nucleotide can be regarded as a phosphoester of a nucleoside. In turn, a nucleoside is a N-glycoside in which the sugar component is ribose or deoxyribose and aglucon is a pyrimidine or purine base. Thus, nucleotide is composed of three units : a phosphate group derived from phosphoric acid (H_3PO_4), attached to a pentose sugar (5 carbons), attached to a nitrogenous base (either a pyrimidine or purine).

The general structure of single nucleotide is shown diagrammatically in Fig. 11.1.

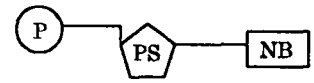


Fig. 11.1 Diagrammatic representation of a nucleotide. P = phosphate groups, PS = pentose sugar and NB = nitrogenous base.

A nucleotide is formed by the reaction of the sugar portion of a nucleoside with phosphoric acid through dehydration synthesis.

$$\text{Nucleoside} + \text{Phosphoric acid} \longrightarrow \text{Nucleotide} + H_2O$$

Nucleotides may be called acids (adenylic acid, guanylic acid and so forth) because their phosphate groups produce hydrogen ions or they may be designated as the specific nucleoside phosphate (adenosine phosphate, guanosine phosphate, and so forth). The names of some nucleotides are listed in Table. 11.1.

The four nucelotides found in DNA are combinations of adenine, guanine, cytosine or thymine with deoxyribose and phosphate.

The four nucleotides found in RNA are combinations of adenine, guanine, cytosine or uracil with ribose and phosphate.

Nucleosides : A nucleoside is a chemical combination of a pentose sugar and pyrimidine or a purine base. The attachment of the two components is a general dehydration synthesis reaction in which a molecule of water is removed from between the base and the sugar.

$$\text{Nitrogenous base} + \text{Pentose sugar} \longrightarrow \text{Nucleoside} + H_2O$$

Thus, a nucleoside is a N-glycoside in which the sugar component is ribose or deoxyribose and the aglucone is a pyrimidine or purine base.

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Table 11.1
NAMES OF NUCLEOTIDES

Adenosine-2′-phosphate	
Adenosine-3′-phosphate	Deoxyadenosine-3′-phosphate
Adenosine-5′-phosphate	Deoxyadenosine-5′-phosphate
Guanosine-2′-phosphate	
Guanosine-3′-phosphate	Deoxyguanosine-3′-phosphate
Guanosine-5′-phosphate	Deoxyguanosine-5′-phosphate
Cytidine-2′-phosphate	
Cytidine-3′-phosphate	Deoxycitidine-3′-phosphate
Cytidine-5′-phosphate	Deoxycitidine-5′-phosphate
Uridine-2′-phosphate	Deoxyuridine-3′-phosphate
Uridine-3′-phosphate	Deoxyuridine-5′-phosphate
Uridine-5′-phosphate	Thymidine-5′-phosphate

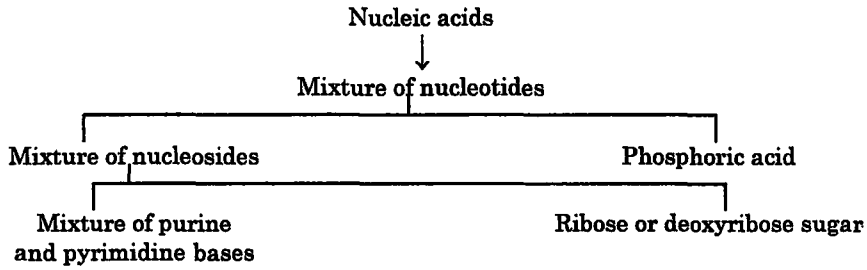
Nucleosides containing ribose are called ribonucleosides. Those containing deoxyribose are called deoxyribonucleosides.

The names of some nucleosides are listed in Table 11.2.

Table 11.2
NAMES OF NUCLEOSIDES

Nucleoside	*Nitrogen base + Pentose sugar*
Adenosine	Adenine and ribose
Deoxyadenosine	Adenine and deoxyribose
Cytidine	Cytosine and ribose
Deoxycytidine	Cytosine and deoxyribose
Uridine	Uracil and ribose
Deoxyuridine	Uracil and deoxyribose
Thymine riboside	Thymine and ribose
Thymidine	Thymine and deoxyribose

The stepwise degradation of nucleic acids will yield different compounds in the following manner—



11.5 Structure of DNA

Introduction : DNA is present in the cells of all plants, animals, prokaryotes and in a number of viruses. In eukaryotes it is combined with proteins to form *nucleoproteins*. In prokaryotes (*e.g., Escherichia coli*, a bacterium) the genetic material consists of a single giant

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molecule of DNA about 1,000 microns in length, without any associated proteins. DNA is present mainly in the chromosomes. It has also been reported in cytoplasmic organelles like mitochondria and chloroplasts. The DNA of all plants and animals and many viruses (polyoma virus, small-pox virus, bacteriophages T2, T4 and T7) is double stranded. In the bacteriophage ϕ X 174, however, it is single stranded. In some viruses the genetic material is RNA. In the tobacco mosaic virus and the bacterial viruses F2 and R17 the RNA is single stranded. In the reovirus and the wound tumour virus, however, RNA is double stranded. In bacteria and in higher plants and animals both DNA and RNA are present. Viruses usually contain either DNA or RNA.

Table 11.3
GENETIC MATERIAL OF ORGANISMS

Double Stranded DNA	Higher animals and plants Bacteria.
(ds DNA)	Polyoma virus and small-pox virus.
	The T-even bacteriophages (T2, T4, T6)
Single Stranded DNA	The bacteriophage $\phi \infty$ 174 and several
(ssDNA)	bacterial viruses.
Double Stranded RNA	Reo group of viruses.
(dsRNA)	Wound tumour virus.
Single Stranded RNA	Tobacco mossic virus.
(ss RNA)	A tobacco virus. Influenza virus. Poliomyelitis virus. Bacterial viruses F2 and R17.

Structure of DNA : The widely accepted molecular model of DNA is the double helix structure proposed by Watson and Crick (1953). The DNA molecule consists of two helically twisted strands connected together by 'steps' (Fig. 11.2 and 11.3). Each strand consists of alternating molecules of *deoxyribose* (a pentose sugar) and *phosphate* groups. Each step is made up of a double ring *purine* base and a single ring *pyrimidine* base. The purine and pyrimidine bases are connected to deoxyribose sugar molecules. The two strands are intertwined in a clockwise direction, *i.e.*, in a right hand helix, and run in opposite directions. The strand completes a turn each 34A. Each nucleotide occupies 3.4A. Thus, there are 10 nucleotides per turn. Each successive nucleotide turns 36 degrees in the horizontal plane. The width of the DNA molecule is 20A. The twisting of the strands results in the formation of *deep* and *shallow spiral grooves.*

The DNA molecule is a *polymer* consisting of several thousand pairs of *nucleotide monomers.* Each nucleotide consists of the pentose sugar *deoxyribose, a phosphate* group and a *nitrogenous base* which may be either a *purine* or a *pyrimidine.* Deoxyribose and a nitrogenous base together form a nucleoside. A *nucleoside* and a phosphate together form a *nucleotide.*

Nucleoside = Deoxyribose + Nitrogenous base.

Nucleotide = Deoxyribose + Nitrogenous base + Phosphate

(1) Deoxyribose is a pentose sugar with five carbon atoms. Four of the five carbon atoms plus a single atom of oxygen form a five-membered ring (Fig. 11.4). The fifth carbon atom is outside the ring and forms a part of a —CH_2 group. The four atoms of the ring are numbered 1′, 2′, 3′ and 4′. The carbon atom of—CH_2 is numbered 5′. There are three—OH groups in position 1′, 3′ and 5′. Hydrogen atoms are attached to carbon atoms 1′, 2′, 3′, and 4′.

Ribose, the pentose sugar of RNA has an identical structure except that there is an —OH group instead of H on carbon atom 2′.

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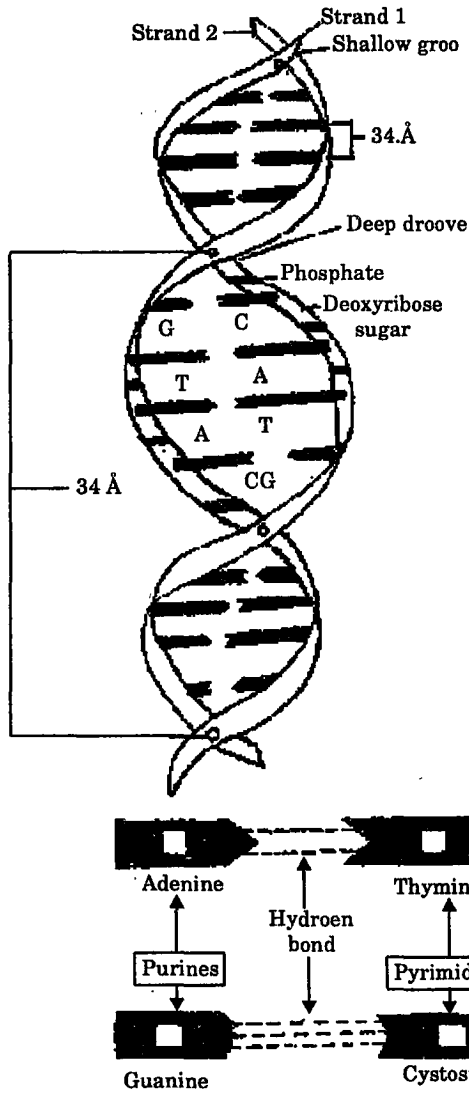

Fig. 11.2. The DNA double helix.

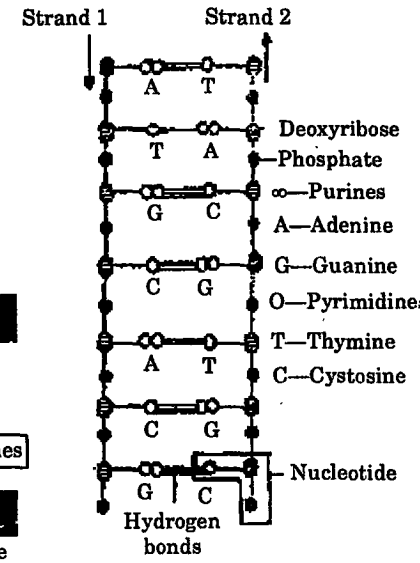

Fig. 11.3. Diagrammatic representation of the DNA molecule.

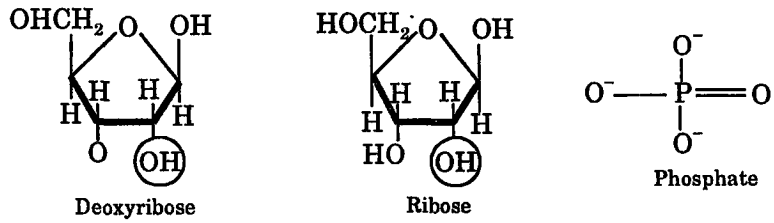

Fig. 11.4. Deoxyribose, ribose and phosphate.

All the sugars in one strand are directed to one end, *i.e.*, the strand has polarity. The sugars of the two strands are directed in opposite directions.

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(2) Nitrogenous bases : There are two types of nitrogenous bases. *pyrimidines and purines.* The pyrimidines are single ring compounds, with nitrogen in positions 1′ and 3′ of a 6-membered benzene ring (Fig. 11.5). The two most common pyrimidines of DNA are *cytosine* (C) and *thymine* (T) (Fig. 11.6). The *purines* are double ring compounds. A purine molecule consists of a 5-membered *imidazole* ring joined to a pyrimidine ring at positions 4′ and 5′. The two most common purines of DNA are *adenine* (A) and *guanine* (G).

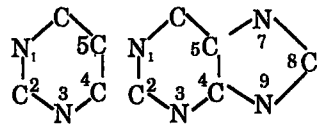

Fig. 11.5. Pyrimidine and purine rings.

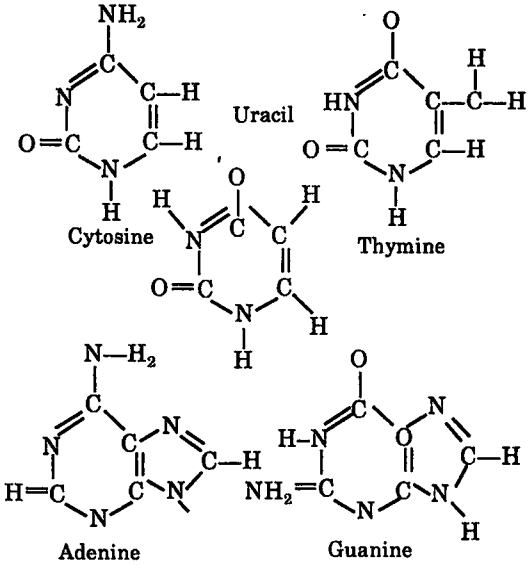

Fig. 11.6. Common nitrogen bases of nucleic acids.

Base pairing (Fig. 11.7 and 11.8). Each 'step' of the DNA ladder is made up of a purine and a pyrimidine pair, *i.e.*, of a double ring and a single ring compound. Two purines would occupy too much space, while two pyrimidines would occupy too little. Because of the purine-pyrimidine pairing the total number of purines in a double-stranded DNA molecule is equal to the total number of pyrimidines. Thus A/T = 1 and G/C = 1 or A + G = C + T. The ratio A + T/G + C, however, rarely equals 1, and varies with different species from 0.4 to 1.9. This ratio is commonly low in micro-organisms and high in higher animals.

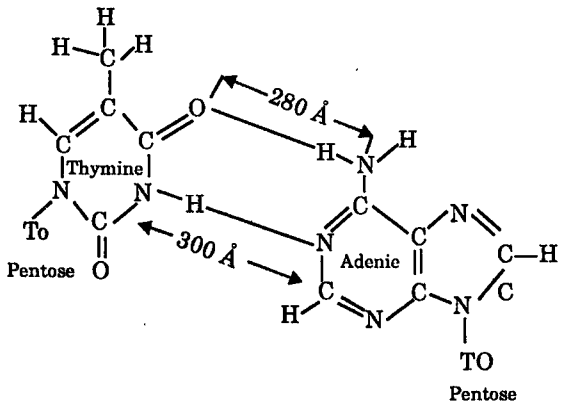

Fig. 11.7A. Pairing between thymine and adenine.

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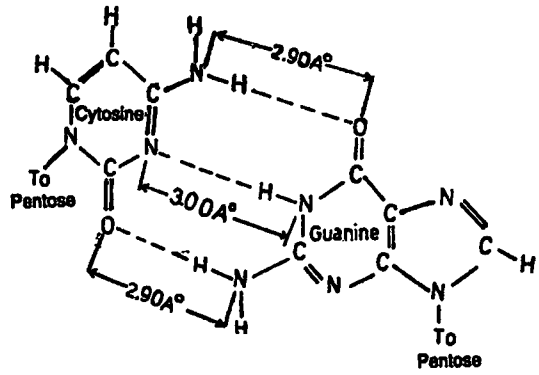

Fig. 11.7 B. Pairing between cytosine and guanine.

The purine and pyrimidine bases pair only in certain combinations. Adenine pairs with thymine (A : T) and guanine with cytosine (G : C). The total width of the pair is 10.7A. Adenine and thymine are joined by two hydrogen bonds through atoms attached to positions 6′ and 1′ (Fig. 11.9). Cytosine and guanine are joined by *three* hydrogen bonds through positions 6′, 1′ and 2′. The hydrogen atom with its positive charge is shared between an oxygen atom and a nitrogen atom, both with slight negative charges. Although hydrogen bonds are weak, the fact that there are so many gives stability to the DNA molecule. The weak hydrogen bonding enables the two strands of the DNA to separate during replication. The pyrimidine and purine bases are linked to the deoxyribose sugar molecules. The linkage in pyrimidine nucleosides is between position 1′ of deoxyribose and 3′ of the pyrimidine. In purine nucleosides it is between position 1′ of deoxyribose and position 9′ of the purine.

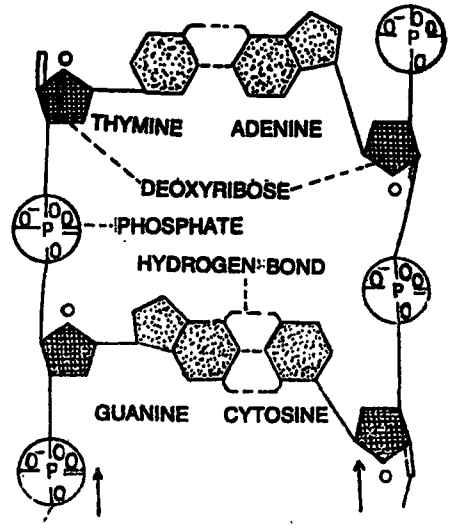

Fig. 11.8. Segment of DNA molecule showing pairing between two nucleotides.

(3) Phosphate : In the DNA strand the phosphate groups alternate with deoxyribose. Each phosphate group is joined to carbon atom 3′ of one deoxyribose and to carbon atom 5′ of other. Thus each strand has a 3′ end and a 5′ end. The two strands are oriented in opposite directions. The 3′ end of one strand corresponds to the 5′ end of the other. Consequently the oxygen atoms of deoxyribose point in opposite directions in the two strands.

Unusual bases in DNA : Although the bases most commonly present in DNA are adenine, guanine, cytosine and thymine, other bases have also been found. In some viruses (*e.g.*, PBS 1

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and PBS 2) uracil occurs in place of thymine in DNA. Also, in some bacterial viruses (bacteriophages) cytosine is replaced by 5-hydroxymethylcytosine (HMC). The variations of C, A and G in DNA can be considered to be the result of methylation of these bases.

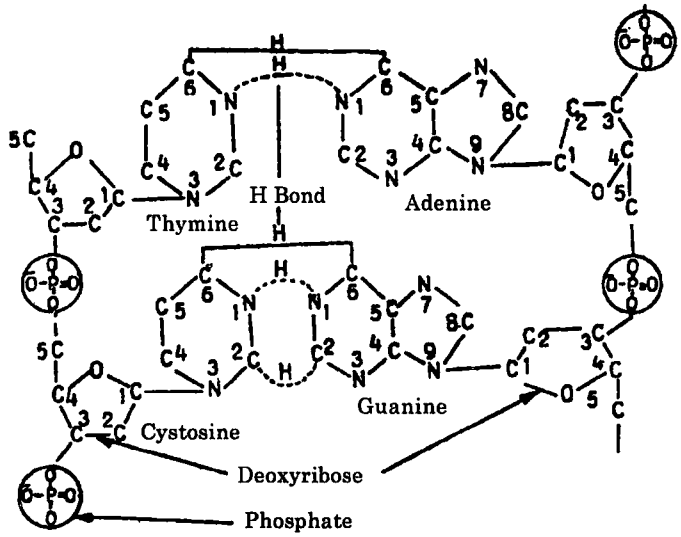

Fig. 11.9. Two pairs of nucleotides showing bonding between phosphate, deoxyribose and nitrogenous bases.

11.6 Single Stranded DNA (Fig. 11.10)

Although DNA of most organisms consists of two strands, single strand DNA is present in the bacterio-phage virus ϕ X174 and in several other bacterial viruses. Single stranded DNA differs from double stranded DNA in the following respects. (1) Ultra-violet absorption of double stranded DNA remains constant from 0 to 80°C, and then rises rapidly (80°C is the critical melting point). In single stranded DNA ultra-violet absorption increases steadily from 20°C to 90°C. (2) Double stranded DNA is resistant to the action of formaldehyde. In single stranded DNA the reactive sites are exposed, and therefore it is not resistant. (3) In double stranded DNA, A = T and G = C. In single stranded DNA of $\phi \times 174$ the proportion of A:T:G:C is 1 : 1.33 : 0.98 : 0.75. (4) Double stranded DNA is linear while single stranded DNA is circular. During replication, single stranded DNA becomes double stranded (replicative form).

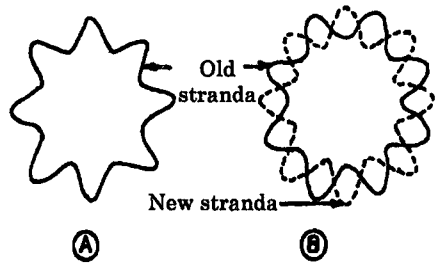

Fig. 11.10. (A) Single stranded DNA. (B) During replication.

11.7 Circular DNA

Most of the organisms have regular double helical DNA. There are some bacteriophages and animal viruses which contain single stranded DNA. However, DNA of most phages is not linear but is in the form of a circle. The DNA of ϕ X174 phages and of polyoma virus, cause cancer in monkey and these are circular. DNA from the mitochondria of higher organisms is also circular.

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The *in vitro* origin of circular DNA in the phage has been investigated. Actually DNA isolated from some viruses is linear. On heating this, the two strands of DNA separate and get denatured. If recooling is done under controlled manner, this brings the two strands together and some normal double stranded DNA is obtained. In this recooling some portion of DNA may take circular form also (Fig. 11.11) probably because the 'sticky' ends of DNA get exposed and the two ends on joining form a circle. By observing under electron microscope, it is found that about 20% of the renatured DNA in polyoma virus takes circular shape during renaturation.

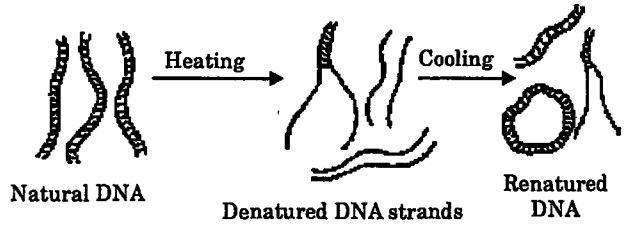

Fig. 11.11. In vitro origin of circular DNA in bacteriophages.

11.8 Structure of Ribose Nucleic Acids

It is also a polynucleotide but the pentose ribose has a free hydroxyl group in position 2′. It is a long chain polynucleotide which does not exist in a regular conformation like a double-chain DNA although some viruses (*e.g.*, reo viruses and wound tumour virus) have double stranded RNA. The single RNA. strand is folded upon itself, either entirely or in certain regions. In the folded region a majority of the bases are complementary and are joined by hydrogen bonds. This helps in the stability of the molecule. In the unfolded region the bases have no complements. Due to this, RNA is not having the purine-pyrimidine equality that is found in DNA. It is understandable that on a single chain or RNA the molar proportion of purines and pyrimidines can vary considerably.

RNA does not contain the pyrimidine base *thymine* but the pyrimidine uracil instead. In regions where purine-pyrimidine pairing takes place, adenine pairs with uracil and guanine with cytosine. This base pairing is of importance when RNA is being synthesised by DNA and when RNA is involved in protein synthesis.

In addition to the four bases mentioned above, RNA has also some unusual bases. There are more unusual bases in RNA than in DNA. All normal RNA chains either start with adenine or guanine.

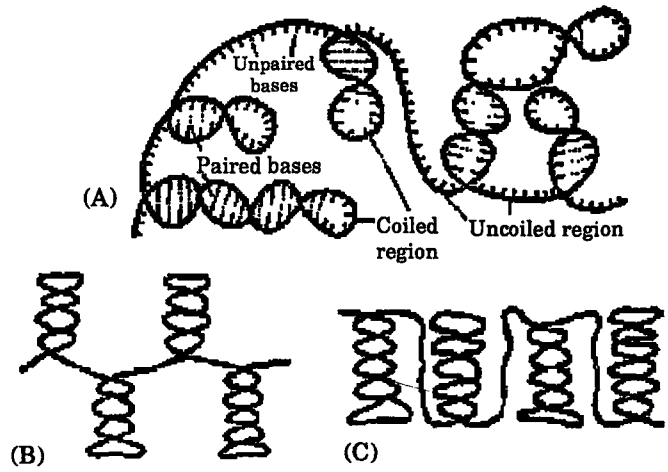

Fig. 11.12. Secondary structure of ribosomal RNA (schematic) (A) As an extended strand; (B) With absence of positive interaction there is mutual repulsion of helical region; (C) With presence of positive interaction there is association between helical regions.

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Types of RNA : There are three distinct RNA species : *messenger* (*m*RNA) or *template RNA, ribosomal* RNA (rRNA) and *soluble RNA* (*s*RNA) or *transfer RNA* (*t*RNA). We will discuss these one by one.

(*a*) *Ribosomal RNA (rRNA) :* It occurs in combination with protein as ribonucleoprotein in the minute round particles called ribosomes which are attached to the surfaces of intra-cellular membrane system called endoplasmic reticulum. It constitutes about 80% of the total RNA of the cell.

It is being synthesised on special regions of chromosomal DNA that are concentrated in the nucleoli, small densely staining spots in the nucleus.

Ribosomal RNA molecule may be a short compact rod, a compact coil or an extended strand (Fig. 11.12).

The *r*RNA does not show pyrimidine equality. The *r*RNA strands unfold upon heating and refold upon cooling. The *r*RNA has been found to be stable for at least two generations.

Depending on the basis of sedimentation and molecular weight there are three types of ribosomal RNA (Table 11.4).

Table 11.4
TYPES OF rRNA

Type of rRNA	*Molecular weight*	*Number of nucleotides*	*Type*
High MW *r*RNA	>million	3,000—5,500	21—29 S
High MW *r*RNA	<million	1,200—2,500	12—18 S
Low MW *r*RNA	~ 40,000	~ 120	5 S

(*b*) *Messenger RNA (mRNA) :* This is so named because it is the type of RNA which carries information for protein synthesis from the DNA (genes) to the sites of protein formation (ribosomes). Only about 5% of total cellular content of RNA is *m*RNA. Its strands exhibit considerable differences in length with molecular masses of about 500,000 to 4 million. Its sedimentation coefficient is 85.

There is evidence that the half-life of *m*RNA may vary from very short to very long. For example, a variety of bacterial *m*RNA is enzymatically broken down within a few minutes of its appearance in the cell.

Messenger RNA is always single stranded. It contains mostly the bases adenine, guanine, cytosine and uracil. The sequence of bases in *m*RNA molecules is complementary to the bases that constitute the genetic code.

In messenger RNA, no base pairing takes place. In fact base pairing in the *m*RNA destroys its biological activity.

It is interesting to note that each gene transcribes its own *m*RNA. Thus, there are approximately as many types of *m*RNA molecules as are genes. These may be 1,000 to 10,000 different species of *m*RNA in a cell. These *m*RNA types differ only in the sequence of their bases and in their length.

Messenger RNA is transcribed on a DNA strand through the enzymatic action of RNA polymerase. Synthesis begins at the 5′ end and proceeds to the 3′ end.

(*c*) *Transfer RNA (tRNA) :* The name transfer RNA is applied to some soluble RNA because of its role in the "transfer" of amino acids in the process of protein synthesis. It is smallest of the RNA species containing about 15 to 80 nucleotides, with a molecular weight of 25,000 *d*. This constitutes 10 to 15% of total RNA of the cell.

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The structure of transfer RNA molecular is conventionally represented in the form of a clover leaf (Fig. 11.13), although recent evidence indicates the *t*RNA molecules are L-shaped.

The structure of alanine transfer RNA has been unravelled by Robert W. Holley and his associates. It consists of a single polynucleotide chain of 77 subunits.

Transfer RNA is synthesised in the nucleus on the DNA template. Transfer RNA does not show any obvious base relationship to DNA.

The main function of transfer RNA is to carry amino acids to *m*RNA during protein synthesis. Each amino acid is carried by a specific *t*RNA.

The transfer RNA molecule has four recognition sites (Fig. 11.13).

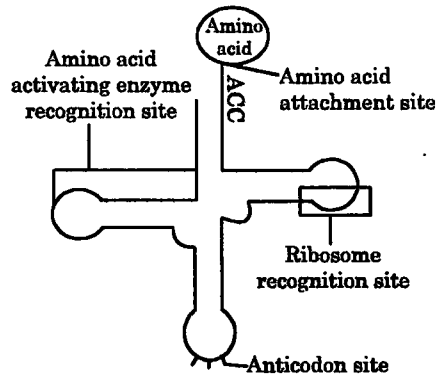

Fig. 11.13. Structure of *t*RNA molecule is shown in the form of a clover leaf : this also shows recognition sites.

(i) Amino acid attachment site : It is the 3′ terminal—CCA sequence.

(ii) Anticodon site : It consists of the middle three bases on the anticodon loop which forms the *anticodon* : The latter recognises the three complementary bases which form the codon of *m*RNA.

(iii) Ribosome recognition site : This is common to all *tRNA* and consists of G—T—ψ—C—R sequence on the T ψ C loop.

(iv) Amino acid activating enzyme recognition site : This is the site by which the activating enzyme recognises and charges specific amino acids with *t*RNA.

Unusual bases : In addition to the usual bases A, U, G and C, tRNA contains a number of unusual bases, and in this respect differs from *m*RNA and *r*RNA. The unusual bases of *t*RNA account for 15-20% of the total RNA of the cell. Most of the unusual bases are formed by *methylation* (addition of–CH_3 or methyl group to the usual bases), *e.g.*, cytosine and guanine on methylation yield *methylcytosine* and *methylguanine*, respectively. Precursor tRNA molecules transcribed on the DNA template contain the usual bases. These are then modified to unusual bases. The unusual bases are important because they protect the *t*RNA molecule against degradation by RNase. This protection is necessary because RNA is found floating freely in the cell.

Some of the unusual bases of *t*RNA are *methylguanine (GMe), dimethylguanine (*GMe_2*)* methylcytosine (Me), *ribothymine* (T), *pseudouridine* (ψ), *dihydrouridine* (DHU, H_2U, UH_2), inosine (I) and *methylinosine* (IMe, MeI). In general, organisms high in the evolutionary scale contain more modified bases than lower organisms.

Table 11.5
COMPARISON OF THE DIFFERENT TYPES OF RNA

	Ribosomal RNA (rRNA)	*Messenger RNA (mRNA)*	*Transfer RNA (tRNA)*
Percentage of total RNA of cell	~80%	3 to 5%	10 to 20%
Sedimentation co-efficient	28S, 18S, 5.8S, 5S 23S, 16S, 5S	8S	3.8S
Number of nucleotides	5S RNA : 120 nucleotides.	E. coli : 900 to 1,500 nucleotides.	73 to 93 nucleotides.

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	16-18S RNA : 1,600 to 2,500 nucleotides. 23-28S RNA : 3,200 to 5,500 nucleotides.		
Molecular weight	23S RNA: 11×10^6 30S RNA : 0.55×10^6	500,000	25,00—30,000.
Unusual bases	Small amount of methylated bases. (E. coli : 1 per 100-150 nucleotides).	Small amount of unusual bases.	High content of unusual bases. (E.coli : 1 per 30-40 nucleotides).
Site of synthesis	Derived from nucleolar DNA.	Synthesized in nucleus on DNA template	Synthesized in nucleus on DNA template.
Beginning of synthesis	Synthesis begins at gastrulation, and increases as development proceeds.	Some mRNA is found in the ovum. New mRNA is synthesized during early cleavage.	tRNA synthesis occurs at the end of cleavage stages.
Base of relationship DNA	No obvious base relationship to DNA. rRNA is formed from only small sections of DNA.	mRNA shows base relationship to DNA. It is formed from all sections of DNA.	Same as in rRNA.
Function	Unpaired bases may bind mRNA and tRNA to ribosomes.	Conveys genetic information from DNA of chromosomes to the ribosomes, where it takes part in protein synthesis.	Adaptor for attaching amino acids to mRNA template.

11.9 Differences Between DNA and RNA

RNA also differs from DNA in having *ribose* as the sugar instead of deoxyribose (Table 11.6).

Table 11.6
COMPARISON BETWEEN DNA AND RNA

DNA	*RNA*
1. DNA is the usual genetic material.	1. RNA is the genetic material of some viruses.
2. DNA is usually *double-stranded.* (In certain viruses DNA is single stranded, *e.g.*, $\phi \times 174$).	2. Most cellular RNA is *single stranded.* (Some viruses *e.g.*, reovirus, have double stranded RNA).
3. The pentose sugar is deoxyribose.	3. The pentose sugar is ribose.
4. The common organic bases are adenine, guanine, cytosine and *thymine.*	4. The common organic bases are adenine, guanine, cytosine and *uracil.*
5. Base pairing : adenine pairs with thymine and guanine with cytosine.	5. Adenine pairs with uracil and guanine with cytosine.
6. Pairing of bases is throughout the length of the molecule.	6. Pairing of bases is only in the helical region.
7. There are *fewer uncommon bases.*	7. There are more *uncommon bases.*
8. DNA is only of *one type.*	8. There are *three types of RNA,* messenger, ribosomal and transfer *RNA.*

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9. Most of the DNA is found in the chromosomes. Some DNA is also found in the cytoplasm, *e.g.*, in mitochondria and chloroplasts.	9. Messenger RNA is formed on the chromosomes, and is found in the nucleolus and cytoplasm. rRNA and tRNA are also formed on the chromosomes, and are found in cytoplasm.
10. Denaturation (melting) is partially reversible only under certain conditions of slow cooling (renaturation).	10. Complete and practically instantaneous reversibility of the process.
11. Sharp, narrow temperature interval of transition in melting.	11. Broad temperature interval of transition in melting.
12. DNA on replication forms DNA, and on transcription forms RNA.	12. Usually RNA does not replicate or transcribe. (In certain viruses RNA can synthesize a RNA chain).
13. Genetic messages are usually encoded in DNA.	13. The usual function of RNA is translating messages encoded in DNA into proteins.
14. DNA consists of a large number of nucleotides, up to 4.3 million.	14. RNA consists of fewer nucleotides, up to 12,000.

Size of DNA Molecules

In viruses and prokaryotes, the total genome* specifying the virus or cell is usually encompassed in a single DNA molecule (the RNA viruses are an exception to this rule). In eukaryotes, including the unicells such as algae, yeast, and protozoa, the DNA is partitioned into a number of chromosomes, each of which is believed to contain a single gigantic DNA molecule. Table 11.7 shows the molecular weights, *M*, of the individual DNA molecules of various organisms. The values for many phages, viral, and bacterial DNA molecules are very accurately known (within 2 percent and, in some cases, exactly); the uncertainty in the values for eukaryotic DNA molecules is ± 50 percent in some cases. Note that the range of size is very great among the viruses and phages but much less so for bacteria.

Table 11.7
SIZES OF VARIOUS DNA MOLECULES

Organism or Particle	*Molecular weight, M*	*Organism or Particle*	*Molecular weight, M*
15 plasmid*	1.4×10^6	Vaccinia virus	121×10^6
Polyoma virus	3.2×10^6	Fowlpox virus	178×10^6
Phage 186*	18×10^6	F' 450 dimer plasmid*	210×10^6
Phage T7*	25×10^6	*Mycoplasm homina*	5.3×10^8
Phage λ*	32×10^6	Most bacteria	$2.0 - 2.6 \times 10^9$
F plasmid*	62×10^6	Yeast	6×10^8
F' *lac* plasmid*	95×10^6	*Drosophila* (fruit fly)	7.9×10^{10}
Phage T4*	106×10^6	Human	8×10^{10}

Note : Phages and plasmids marked with an asterisk have *E.coli* as a host. *Mycoplasm homina* is the smallest known bacterium. For yeast, *Drosophila*, and humans the molecular weight of the largest DNA molecule in the organism is given.

*The *genome* is the total DNA content of a haploid organism or, for a diploid organism, the DNA that contains one complete set of genes.

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The length of these molecules can be obtained from this relation : 1 micrometre (μm) = 2×10^6 molecular weight units. Thus, the molecules listed in the table range from 0.7 μm to 40,000 μm (4 cm!). The width of a DNA molecule is 20 Å = 0.002 μm.

In general, the genomes of the more complex organisms require much more DNA than for simpler organisms (though the cells of both the toad and the South American lungfish have considerably more DNA than human cells).

The great length of DNA molecules makes them extremely susceptible to breakage by the hydrodynamic shear forces resulting from such ordinary operations as pipetting, pouring, and mixing. Unbroken DNA molecules for which $M < 2 \times 10^8$ can usually be isolated with ease from phages and viruses. However, when DNA is isolated from bacteria and higher organisms, unless great care is taken, the DNA molecules are almost always broken. In fact, the mean value of M for a sample is usually about 25×10^6, so bacterial DNA, for instance, is frequently fragmented into about a hundred pieces. For plant and animal cells the yield of unbroken molecules in the least broken samples is usually a small fraction of 1 percent. There are several instances in which the fact that the DNA of bacteria and of higher organisms is invariably fragmented by manipulation has important experimental consequences.

Denaturation

The free energies of the weaker non-covalent interactions are not much greater than the energy of thermal motion at room temperature; thus, at elevated temperatures the three-dimensional structures of both proteins and nucleic acids are disrupted. A macromolecule in a disrupted state, in which the molecules are in a nearly random coil conformation, is said to be *denatured;* the ordered state, which is presumably that originally present in nature, is called *native.* A transition from the native to the denatured state is called *denaturation.* When double-stranded DNA or native DNA is heated, the bonding forces between the strands are disrupted and the two strands separate; thus, *denatured DNA is single-stranded.*

A great deal of information about structure and stabilizing interactions has been obtained by studying nucleic acid denaturation. This is done by measuring some property of the molecule that changes as denaturation proceeds—for example, the absorption of ultraviolet light. Originally, denaturation was always accomplished by heating a DNA solution, so a graph of a varying property as a function of temperature is called a *melting curve.* Reagents are known that either break hydrogen bonds or weaken hydrophobic interactions. These are powerful denaturants. Thus, denaturation is also studied by varying the concentration of a denaturant at constant temperature. In the case of DNA, the simplest way to detect denaturation is to monitor the ability of DNA in a solution to absorb ultraviolet light whose wavelength is 260 nanometres (nm). The bases of nucleic acids absorb 260-nm light strongly. A convenient measure of the absorption is the absorbance (A) of a solution—that is, $-\log_{10}$ [(intensity of light transmitted by a solution 1 cm thick)/(intensity of incident light)]. The absorbance at 260 nm, A_{260}, is proportional to concentration, with a value of 0.02 units per microgram DNA per millilitre. More important, the amount of light absorbed by nucleic acids is dependent on the structure of the molecule. The more ordered the structure, the less light is absorbed. Therefore, free nucleotides absorb more light than a single-stranded polymer of DNA or RNA and these in turn absorb more light than a double-stranded DNA molecule. For example, three solutions of double-stranded DNA, single-stranded DNA, and free bases, each at 50 μg/ml, have the following A_{260} values :

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Double-stranded DNA	$A_{260} = 1.00$
Single-stranded DNA	$AK_{260} = 1.37$
Free bases	$A_{260} = 1.60$

This relation is often described by stating either that double-stranded DNA is *hypochromic* or that the bases are *hyperchromic.*

If a DNA solution is slowly heated and the A_{260} is measured at various temperatures, a melting curve such as that shown in Figure 11.14 is obtained. The following features of this curve should be noted :

1. The A_{260} remains constant up to temperatures well above those encountered by living cells in nature.
2. The rise in A_{260} occurs over a range of 6—8°C
3. The maximum A_{260} is about 37 percent higher than the starting value.

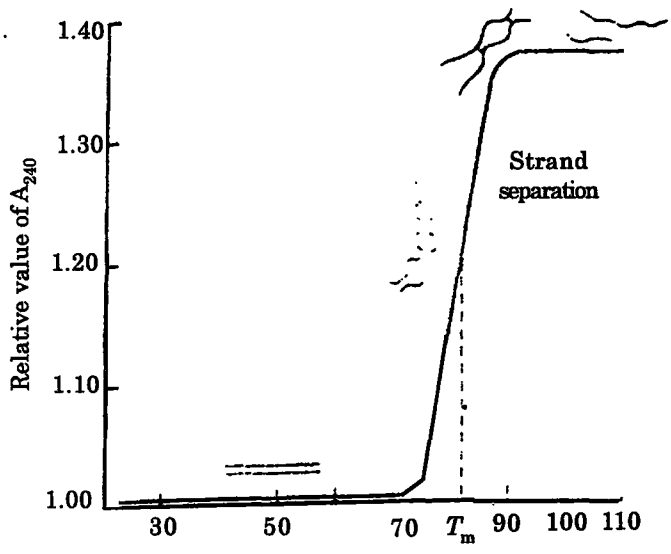

Fig. 11.14. A melting curve of DNA showing T_m and possible molecular confirmations for various degrees of melting.

The state of a DNA molecule in different regions of the melting curve is also shown in the figure. Before the rise begins, the molecule is fully double-stranded. In the rise region, base pairs in various segments of the molecule are broken; the number of broken base pairs increases with temperature. In the initial part of the upper plateau a few base pairs remain to hold the two strands together until a critical temperature is reached at which the last base pair breaks and the strands separate completely.

A convenient parameter to characterize a melting transition is the temperature at which the rise in A_{260} is half complete. This temperature is called the melting *temperature;* it is designated T_m.

Renaturation

A solution of denatured DNA can be treated in such a way that native DNA re-forms. The process is called *renaturation or reannealing* and the reformed DNA is called *renatured DNA.*

Renaturation has proved to be a valuable tool in molecular biology since it can be used to demonstrate genetic relatedness between different organisms, to detect particular species of RNA, to determine whether certain sequences occur more than once in the DNA of a particular organism, and to locate specific base sequences in a DNA molecule.

Requirements for Renaturation

Two requirements must be met for renaturation to occur :

1. The salt concentration must be high enough that electrostatic repulsion between the phosphates in the two strands is eliminated—usually 0.15 to 0.50 *M* NaCl is used.

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2. The temperature must be high enough to disrupt the random, intra-strand hydrogen bonds. However, the temperature cannot be too high, or stable interstrand base-pairing will not occur. The optimal temperature for renaturation is 20 – 25° below the value of T_m.

Renaturation is a slow process compared to denaturation. The rate-limiting step is not the actual rewinding of the helix (which occurs in roughly the same time as unwinding) but the precise collision between complementary strands such that base pairs are formed at the correct positions. Since this is a result only of random motion, it is a concentration-dependent process; at concentrations normally encountered in the laboratory this takes several hours.

Mechanism of Renaturation

The molecular details of renaturation can be understood by referring to the hypothetical molecule shown below, having a sequence that is repeated several times.

IA	IB	II	IC
. . . ATGA. . .	ATGA. . .	CCCC. . .	ATGA . . .
. . . TACT. . .	TACT. . .	GGGG. . .	TACT . . .
IA′	IB′	II′	IC′

Assume that each single strand contains 50,000 bases, and that the base sequences are complementary. However, any short sequence of bases (say, 4 – 6 bases long) will certainly appear many times in such a molecule and can provide sites for base-pairing. Random collision between non-complementary sequences such as IA and II′ will be ineffective but a collision between IA and IC′ will result in base-pairing. However, this will be short-lived, because the bases surrounding these short complementary tracts are not able to pair and stacking stabilization will not occur. Thus, at the elevated temperatures that have brought about strand separation, these paired regions rapidly become disrupted. However, as soon as two sequences such as IB and IB′ pair, the adjacent bases will also rapidly pair and the entire double-stranded DNA molecule will "zip up" in a few seconds.

It is important to realize that each renatured native DNA molecule is not formed from its own original single strands : in a solution of denatured DNA, the single strands freely mix so that during renaturation strands join at random. This was shown in an experiment using two DNA samples isolated from *E. coli* grown, in one case, in a medium containing $^{14}NH_4Cl$ and in the other, $^{15}NH_4Cl$. The two DNA samples were mixed, denatured, renatured and centrifuged to equilibrium in a CsCl density gradient. The result was a mixture containing three types of native DNA molecules—25 percent contained ^{14}N in both strands, 50 percent contained ^{14}N in one strand and ^{15}N in the other, and 25 percent contained ^{15}N in both strands, which indicates random mixing of the strands during renaturation. This type of molecular mixing is called *hybridization*.

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12 Plant Growth Substances

12.1 Introduction

It has been well established that the growth of one part of a plant tends to influence the growth and development of other parts. It was J. Sachs in 1880 who reported that a chemical substance is able to coordinate growth among different plant parts or different physiological processes. This chemical substance was termed as *hormone or phytohormone.*

Plant hormones, differ in certain respects from the concept of animal hormones. In case of plant hormones we cannot always differentiate between the site of hormone synthesis and place of action, although there are evidences indicating their effects at sites away from the place of synthesis. Another difference is that the effects of animal hormones are rather specific, a plant hormone, however, can elicit a variety of responses depending upon the type of organ or tissue in which it is acting. For these reasons plant hormones have been referred to as "plant growth regulators" or "plant growth substances" or "growth hormones." According to Thimann (1948, '52) a hormone may be defined as *"an organic substance produced naturally in higher plants, controlling growth or other physiological functions at a site remote from its place of production and active in minute amounts."*

The main naturally occurring plant growth regulators are auxins, gibberellins, cytokinins, ethylene and abscisic acid (ABA). The first four are called plant hormones while ABA is naturally occurring growth inhibitor. In plants, these substances occur in small quantities and are able to control many of the physiological processes involved in plant development.

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Finally, there is ethylene which inhibits growth and has other effects such as promotion of ripening.

In addition to these several dozen other plant hormones have been proposed. Some of them are well established, others may be found to be of little importance. Several remain hypothetical because they have never been extracted from plants and indentified chemically.

Several of the proposed hormones have been involved in reproductive development. One is *vernalin* which plays a role in the low temperature preconditioning that makes biennials to grow tall and bloom in the second year of their life.

Perhaps the most extensively investigated reproductive hormone of plants is florigen which was proposed by a Russian botanist in 1936. The hormone is supposed to be produced in leaves and is transported to buds, making them to develop into flower buds rather than leaf buds. Although several experiments provide support for the florigen concept, but florigen has never been extracted definitely from plants and also has not been identified chemically. It thus remains a hypothetical hormone.

Table 12.1

CLASSIFICATION OF PLANT HORMONES

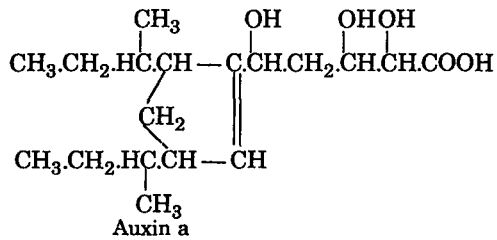
NATURAL		*ARTIFICIAL*	*POSTULATED*
Indole hormone	Non-indole hormone	1. IPA	1. Rhizocaline
A. Auxin		2. IBA	2. Caulocaline
1. IAA		3. NAA	3. Phyllocaline
2. Indole acetonitrile		4. 2, 4-D	4. Florigen
3. Indole acetaldehyde		5. 2, 4, 5-T	5. Vernalin
			6. Dormin
	Nitrogenous	Non-nitrogenous	
B. Glucobrassicin	1. Cytokinins	1. Gibberellins	

We shall now describe important plant hormones one by one.

I. Auxins

Among the growth hormones, auxins were the first to be discovered and studied in detail. In 1928, Went demonstrated its effect on plant growth by performing some experiments with the oat coleoptile.

In 1933, Kogl *et al.* reported the isolation of two compounds *auxin a* (from human urine) and *auxin b* (from maize green oil) which were regarded to be active in regulating the plant growth. These were assigned the following structures.



Auxin a

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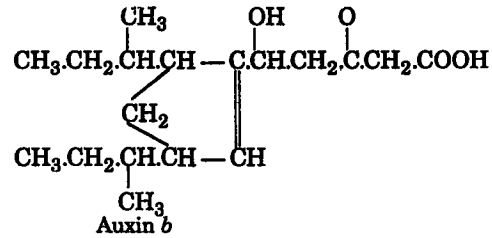
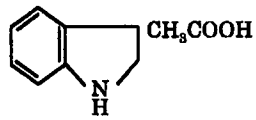


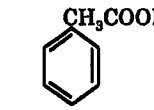
Auxin *b*

It is interesting to note that both auxin *a* and auxin *b* could not be isolated from the growing plant tissues. Therefore, these compounds are of historical importance only.

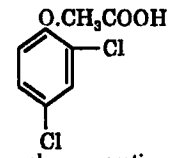
In 1934, Kogl isolated another substance which was identical to indole 3-acetic acid. It is now well established that indole 3-acetic acid is the natural common plant hormone and not auxin *a* and auxin *b*. Later on, it was also found that a number of derivatives of indole-3-acetic acid are also very active. Further, it was also found that a number of derivatives of aryl acetic acid and aryloxy acetic acid are also active, *e.g.*, phenyl acetic acid, 2-4-dichlorophenoxyacetic acid (2, 4D), α-naphthalene acetic acid and β-naphthoxy acetic acid.



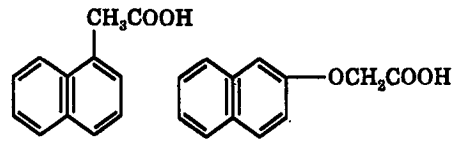
Indole 3-acetic acid



Aryl acetic acid



2, 4-Dichlorophenoxy acetic acid (2, 4D)



α-Naphthalene acetic acid

β-Naphthoxy acetic acid

There are several synthetic auxins which are used commercially for the production of roots on stem cuttings. Examples of these are indole propionic acid, indole butyric acid, naphthyl acetic acid, phenyl acetic acid and phenoxy acetic acid.

Site of production : Auxins are synthesised in the shoot tips, young leaf prinordia, developing seeds and from where they are migrated to the regions of elongation.

Bioassay : Bioassay of auxins is carried out by observing its effect on curvature of oats coleoptile.

The experiment to demonstrate that auxin is synthesised in the coleoptile tip and is translocated downward was demonstrated by Went in 1928 who performed some experiments with the oat coleoptile (Fig. 12.1). In Fig. 12.1, it can be seen that :

(*i*) *1a* is a oat coleoptile with its tip intact. After few hours it showed growth and is indicated by *1b*.

(*ii*) If the tips of coleoptile were removed (represented by 12.1(2a), no growth took place which is shown by *2b*.

(*iii*) If the freshly cut coleoptiles were kept in contact with agar blocks for a few hours (during this period auxin diffused into the agar block) and then the agar blocks were kept on

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the cut ends on the coleoptiles [Fig. 12.1(3*a*) and (4*a*)], growth occurred [Fig. 12.1 (3*b*) and (4*b*)]. If an impregnated agar block was kept laterally on the cut tip of a coleoptile [Fig. 12.1(4*a*)], only that side of the coleoptile got elongated, causing a curvature.

The above experiments clearly demonstrated that auxin is synthesised in the coleoptile tip and is translocated downward.

Different parts of the plants respond differently to the concentration of auxin (shown in Fig. 12.2).

From Fig. 12.2, it is evident that :

(*i*) In case of roots, low concentrations of auxin will stimulate root growth. But if there occurs any increase in the concentration of auxin any more, there occurs the retardation of the growth.

(*ii*) In Fig. 12.2 it is evident that the concentrations which increase stem growth retard the root growth. High concentrations of auxins also retard stem growth.

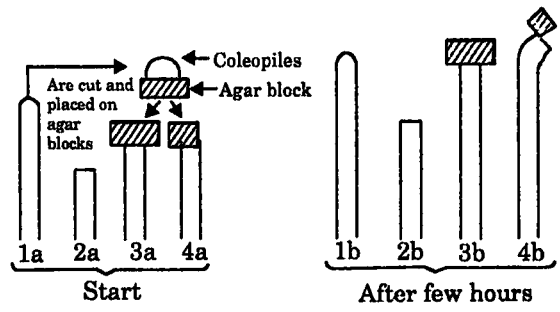

Fig. 12.1. Oat coleoptile

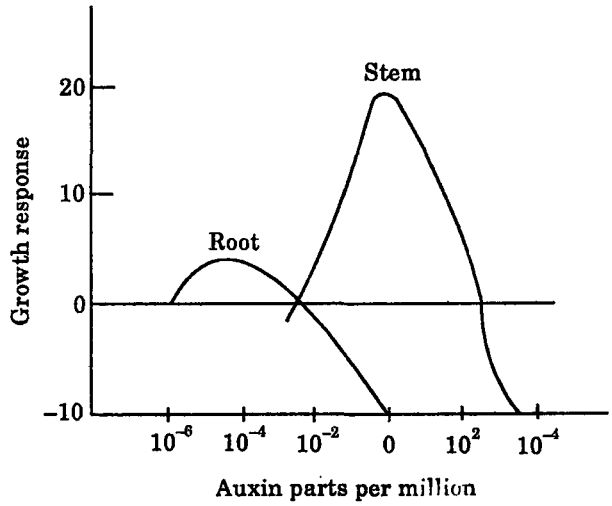

Fig. 12.2. Root and stem responses to varying concentrations of auxins.

Auxin Biosynthesis

The pathway of auxin synthesis in living plant cells involves a series of steps, all of which originate from the amino acid tryptophan, a compound with an indole nucleus which is universally present in plant tissues (Wildman *et al.*, 1947). The general path in the synthesis of auxin is the formation of indole pyruvic acid, an intermediate by oxidative deamination. The conversion may involve the formation of tryptamine by decarboxylation. IAA is formed via indole-3-acetaldehyde, where a number of oxidative enzymes may complete the final conversion to indole acetic acid (Gordon, 1961).

IAA may be formed from nitrile compound (indole-3-acetonitrile) by *nitrilase* (Thimann and Mahadevan, 1958). This nitrile compound is formed from glucobrassicin (Gmelin and Virtanen, 1961; '62)

IAA may also be formed from ascorbigen (another glycoside) on hydrolysis (Kutacek *et al.*, 1960). The whole scheme of auxin synthesis is shown in Fig. 12.3.

Tryptophan may be converted to malonyl tryptophan and thus blocking the auxin synthesis (Lenk, 1963).

Synthesis of auxin is mainly affected by the types of tissues, light intensities and by zinc. Deficiency of zinc will prevent auxin formation by retarding the synthesis of tryptophan—the auxin precursor.

Riddle and Mazelis (1964) showed that the synthesis of IAA proceeds according to the following scheme.

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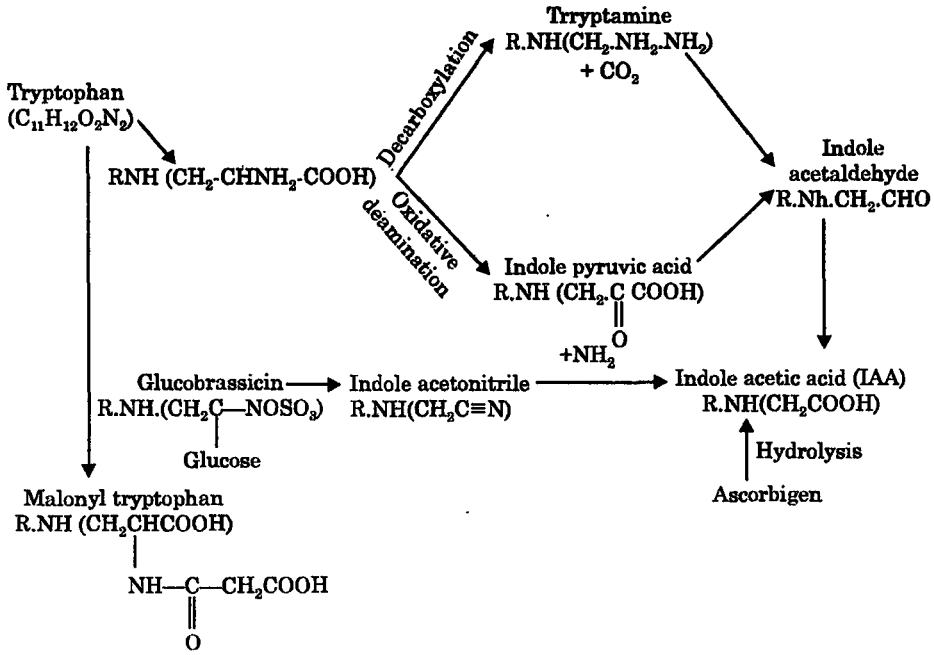

Fig. 12.3. Possible biosynthetic pathway for indole acetic acid (IAA) in plants.

$$\text{Tryptophan} \xrightarrow[Mn^{++} \text{ and Pyridoxal-5-(P)}]{\text{Peroxidase}} \text{Indole-3-acetamide} \rightarrow \text{IAA}$$

Apart from the indole compounds shown in Fig. 12.3 regarding the synthesis of IAA, a number of other indoles are known to occur naturally in plants. It is possible that any one or all of these indoles could serve as precursor of IAA synthesis, but actual details are not known.

Mechanism of auxin action : The problem how a minute amount of hormone (auxin) can bring about such dramatic change in the growth of the plant is one of the interesting and biologically challenging problems in plant physiology.

Several theories have been proposed on the mechanism of hormone action which may be summed up in the following sections :

(a) Hypothesis of osmotic effect :The ultimate effect of growth by cell enlargement is caused due to the enlargement of the protoplast by water uptake. Thus the auxin softens the cell wall by increasing its plasticity (Heyn, 1931; Tagawa and Bonner, 1957; Cleland, 1958) which ensures the swelling of the wall by simple osmotic water uptake.

In 1931, Heyn showed that auxins cause a softening of the cell wall. Since the cell walls are cemented together by pectin compounds made firm by calcium, this Ca-linkage must be removed to ensure the softening of the cell wall and to cause growth. Thus the effect of auxin in removing calcium linkage has been suggested by Bennet-Clark (1956) and the site of the Ca-linkage is the main region of auxin attack. In 1960 Masuda suggested that auxin brings about an increase in the available RNA which binds Ca-ions, thus making them less effective in cementing the cell walls of the plant cells.

Besides cell plasticization auxin can cause the synthesis of new cell wall materials. Thus it has been observed by Christiansen and Thimann (1950) that in pea, auxin treatment causes the accumulation of cellulose and hemi-cellulose. This idea of cell wall synthesis as a basic growth stimulus by auxin is however overturned by Bennet-Clark (1956), Ordin and Bonner (1957) and others.

(b) Hypothesis of enzyme effect : It has been found by various workers that by the addition of hormone there is an increase in the enzymatic activity within the cell.

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Northern (1942) found that hormones caused a decrease in the cytoplasmic viscosity and at the same time brought about dissociation of protein constituents of the cytoplasm. This dissociation activates the enzymes particularly in increasing the availability of the substrate for the enzyme. As a result of this, respiratory activity increases and consequently growth might follow.

Another enzymatic mechanism of auxin action has been proposed by Thimann (1951) who showed that sulphydryl containing enzymes are very much related with growth. According to him the auxin acts not as enzyme activating agent but as agent protecting auxin from inactivation.

(c) Molecular reaction hypothesis : Skoog *et al.* (1942), Foster *et al.* (1962) and many others approached this problem from different points of view and suggested that auxin may act by attaching to some entity (enzymatic) of the cell. Thus according to them auxin acts as a sort of co-enzyme.

Thus the mysterious problem of auxin action still remains unsolved though several hypotheses have been put forward to explain this mechanism. The concept that auxin softens the cell wall which permits easy water uptake and expansion of the cell wall is very attractive. But it is still a matter of controversy as to how this action may be related to changes in pectic or other structural components of the cell wall.

The mode of auxin action have been shown in Fig. 12.4.

Role of Auxins

(a) On growth : The action of auxins in controlling growth appears to be a complex of many functions. Since the early work of Went (1928), a large number of workers in recent years have come to the conclusion that no growth is possible without auxin. The relative sensitivity to auxins of different plant organs has been shown by Boysen-Jensen (1936). According to him roots have a much higher sensitivity to auxin and they can be stimulated to growth at a much lower concentration than stems.

The role of auxins is to both stimulate and inhibit the various growth functions of plants. They can promote or inhibit the differentiation of buds, production of flowers, abscission, activity of certain enzymes etc. and in all these cases, the effect obtained is due to effective auxin action in the tissues.

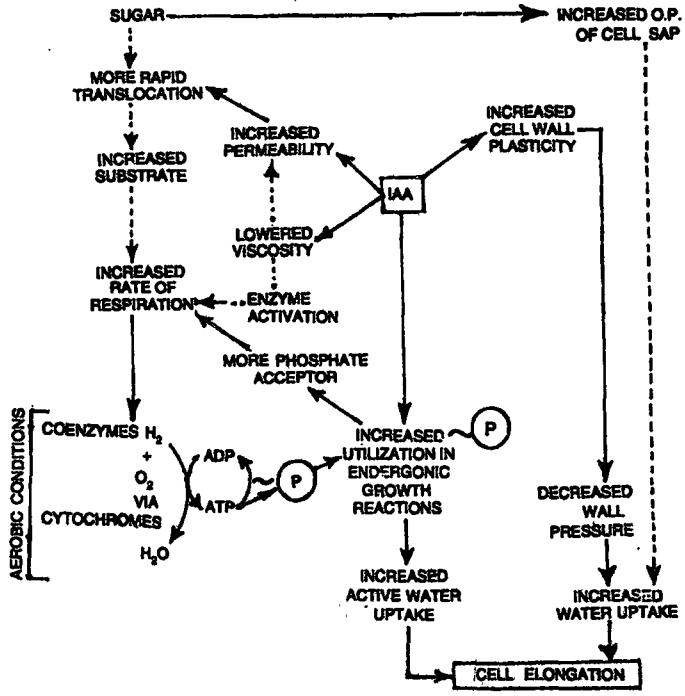

Fig. 12.4. Interrelations between the possible modes of auxin action (modified after Strafford, 1965).

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(*b*) *On tropism and movement* : The effect of auxin in tropism and plant movements has been effectively carried out on the curvature of *Avena* coleoptiles.

It has been independently worked out by Cholodny (1927) and Went (1928) that unequal distribution of auxin was responsible for the bending of plants towards light. The unequal distribution of auxin is due to light-induced lateral distribution of auxin.

Another cause of phototropic curvature is reaction of light on growing cells. Light causes a desensitization of growing cells to a given amount of auxin (Galston and Baker, 1953), but the actual relationship has however not been established.

Other factor of phototropic curvature is the destruction of auxin by light and thus under lighted condition auxin destruction on the lighted side will cause curvature towards the light.

Since the photo-destruction of auxin is a chemical reaction induced by light, it (light) must be absorbed by some plant pigments. According to Wald and DuBois (1936), β-carotene and according to Galston (1949), riboflavin is the photoperiodic pigment responsible for the photo-destruction of auxin.

Like phototropism, auxins have a pronounced effect on geotropism. As in phototropism, geotropism is also due to lateral distribution of auxin by gravitational forces (Dock, 1929). Due to gravitational force nearly 60% of auxin appear on the lower side of root tip or stem. This asymmetrical distribution of auxin causes an asymmetrical growth—stems bending away from the gravitational force and roots towards it (as roots are more sensitive to auxin action at a lower concentration than stems).

Another possible explanation of geotropism has been made by van Overbeek *et al.* (1945), who showed that more auxin formation takes place in meristems just above each node after it has been subjected to geotropic stimulation. So in horizontal position auxin is synthesized on the lower side and consequently more activated.

A similar type of tropic movement (*e.g.*, thigmotropism) has been discussed by Went and Thimann (1937).

(c) Inhibition effect : Thimann and Skoog (1933) first demonstrated that apex is the main source of auxin for the plant and its removal results in the loss of apical dominance and stimulates the development of lateral buds. So the presence of larger amount of auxin in the apex favours the development of an unbranched plant form whereas plants with low auxin concentrations develop a branching habit.

As the auxin concentration in a plant is the primary factor in the inhibition of lateral buds, treatments like irradiation, injection of dyes in the light etc. which reduce the auxin level will stimulate branching (Chailakhyan and Zdanova, 1933; Leopold, 1949).

Branching in roots also involves an auxin mechanism similar to shoots, as decapitation of the roots can cause branching.

(d) Organ differentiation : Auxins and other plant hormones not only affect growth by elongation, but also affect the morphological type of growth. Thus by applying auxins a young stem may develop cluster of cells differentiated into callus, roots, vegetative buds and sometimes even flowers. Like other auxin effects a dualism is exerted in the control of organ differentiation. A relatively high concentration of auxin causes dedifferentiation of tissues which result in the formation of callus. Whereas under certain circumstances, auxin can redifferentiate these callus or meristems into roots, buds or flowers.

(e) Fruit development : The role of auxin in the normal process of fruitset without pollination (parthenocarpic fruit-set) has been thoroughly investigated by Gustafson (1936). This discovery of fruit-set by auxin treatment has a great value in commercial practice in producing fruit-set when natural set is difficult.

To find out the actual cause of fruit-set by auxin treatment is a difficult problem and it might be due to either "incitement of growth of the young fruit" or due to prevention of abscission of the flower.

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(f) Abscission : In most plant species there comes a time during the life of each leaf and fruit when they shed from stem. The process by which leaves and other organs such as fruits and flowers are removed from the plant is known as *abscission*. It is due to formation of separation or *abscission layer* at the base of the petiole. It is like thin plate of cells oriented at right angles to the axis of the petiole. The wall of the separation layer becomes softened and gelatinous, forming a weak region which readily breaks under slight strain.

Formation of separation layer is related to a fall in the auxin content. As the organs set older their auxin content declines and consequence is the formation of separation layer. The use of synthetic hormones in the abscission of leaf has been pointed out by LaRue (1939) and later on its role in the control of abscission of petioles, flowers and fruits has also been pointed out.

The auxin action on abscission has been experimentally proved by Addicott and Lynch (1951). The effect of its action depends on the locus of its application. Auxin applied to the distal cut end of a petiole after removal of auxin-synthesizing lamina delays abscission of the petiole; whereas when it is applied on proximal side (stem) it has an opposite effect, *i.e.*, abscission is accelerated rather than delayed by auxin.

Action : The exact mechanism of action of auxin is not understood clearly.

Auxins are able to enlarge the cells by activating the synthesis of some enzymes which get involved in cell wall metabolism. Auxins have also been able to induce synthesis of cellulose, hemicellulose and pectic materials. It is found that the application of auxins is followed by increase in DNA, m-RNA, r-RNA and protein. It is also accepted that auxins bind with the Ca^{2+} of the cell walls, increase their plasticity and then the cell walls swell by taking in water. This results in the enlargement of the cells.

Natural auxins get synthesized from tryptophan. In higher plants, they are synthesized in the apical parts of the shoots.

Auxin inactivation : Inactivation is almost synonymous with 'destruction,' but it means only the "loss of biological activity in a chemically determined manner." Inactivation of auxin is a normal phenomenon in plant life. Early experiment of Went (1928) with exposure of *Avena coleöptile to light indicates* the reduction in the amount of auxin present. This led Tang and Bonner (1947), cesses—enzymatic and non-enzymatic—can inactivate auxin in plants. Photoactivation of auxin is chiefly due to the presence of photodynamic compounds like riboflavin, methylene blue etc. (Gordon, 1954). The detail account of auxin destruction has been made by Larsen in 1951. The whole scheme has been represented in Fig. 12.5.

In this scheme reduced pigment (flavo-protein complex) is first oxidised by oxygen and forms peroxide (I). Next stage is an enzymatic reaction leading ultimately to the destruction of auxin. The enzyme involved is a *peroxidase*. As a result of this reaction indole acetic acid (auxin) is oxidised to indole aldehyde and carbon dioxide (II). Another alternative reaction of the destruction of IAA is a non-enzymatic one where IAA is oxidised without the participation of an enzyme (IIA). Possibly a second non-enzymatic destruction of auxin proceeds (III) in the generation of the original reduced pigment (flavo-protein).

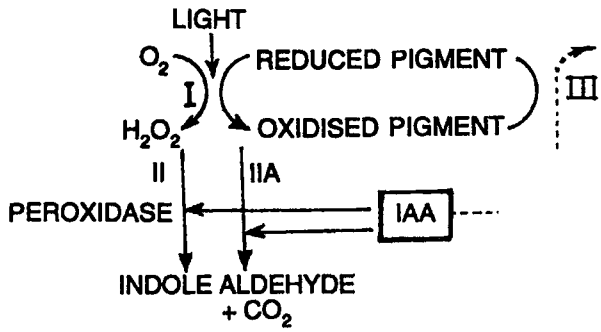

Fig. 12.5. Possible scheme of auxin inactivation.

Regarding the effect of light in the enzymatic reaction Galston and Baker (1941) showed that there is a natural inhibitor in pea and activity of this inhibitor in pea and

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activity of this inhibitor can be overcome by light, causing the destruction of auxin. Light therefore acts to prevent the effect of the inhibitor on the enzymatic destruction of auxin.

The nature of enzyme responsible for auxin destruction is also a matter of dispute. It may be either an iron-enzyme (*cytochrome oxidase*) or a *peroxidase.*

The ultimate products of the auxin destruction are indole aldehyde and carbon dioxide in both the enzymatic (II) and the non-enzymatic (IIA) destruction (Refer Fig. 12.5).

Auxin inactivation in plants may be due to formation of hormonally-inert complexes of various types. Thus IAA can readily be esterified by plant enzymes to form indole ethyl acetate or with amino acid to form indole-acetyl-aspartic acid. IAA also forms conjugates with various sugars and sugar-alcohols.

Auxin destruction can also be made by increasing pH of the medium, X-ray and heat treatment.

12.2 Gibberellins

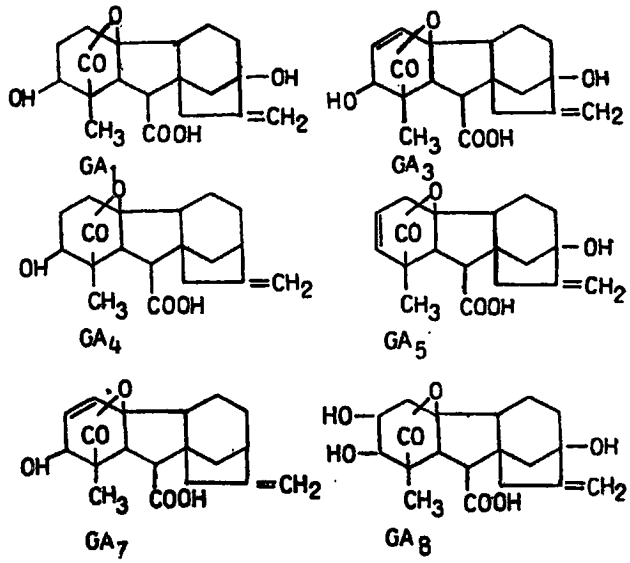
In 1898, it was reported that there occurred anomalous growth of rice seedlings called "bakanae" (meaning stupidly overgrown seedling) disease. In 1928, the disease was correctly attributed to a toxin produced by a fungus which was classified later as *Gibberella fujikuroi (fusarium moniliforme).* In 1938, Yabuta and Sumiki isolated two substances which were named Gibberellins. Their crystals with m.p. 245-246°C (dec), the active substances, were named gibberellin A (1941), but were subsequently found to be a mixture of three components (1955), gibberellins A_1, A_2 and A_3 (the GA_3, the most representative of the gibberellins is also called gibberellic acid). The second substance isolated in 1938 having m.p. 194-196°, gibberellin B, was devoid of activity, and was later found to correspond to allogibberic acid.

About 40 gibberellins have been isolated to date as metabolites of the fungus *G. fujikurol.* Most of these are C_{19} gibberellins. However, some contain a carbon substitutent at C-10 and are called C_{10} gibberellins.

Major structural variations among gibberellins are the presence or absence of a double bond and hydroxyls at C-1, C-2 and of hydroxyls at C-13 and C-16; gibberellin glycosides are also found.

Gibberellins are named with a subscript after A such as GA_1, GA_2, GA_3...

Structures of some common gibberellins are give below :



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Occurrence of gibberellins : Most of the workers (West and Phinney, 1958) believe that gibberellins occur naturally in the plants. According to Corcoran and Phinney (1962), most of the gibberellins occur in maturing seeds particularly involved in the seed maturation processes. Gibberellins are also present in the germinating seedling (Wheeler, 1960) and are said to be abundant in the growing tissues such as expanding cotyledon or leaf.

Gibberellins occur in plants either in bound or modified form.

Biosynthesis of gibberellins : All terpenoids are basically built up from "isoprene units," which are five-carbon compounds. The linking of two isoprene units yields a monoterpene (C = 10). The question of biosynthesis of gibberellins is still a debatable one. However the work of Birch *et al.* (1958) with radioactive isotope suggests a possible pathway that might lead to the synthesis of gibberellins. The possible precursor of the gibberellins is a two carbon compound. acetate. By labelling the carbon atom (^{14}C) of the carboxyl group of the acetate, Birch *et al.* (1958) observed that gibberellic acid (GA_3) is synthesized *via* mevalonate and geranylgeraniol. The whole scheme of the synthesis of gibberellic acid is given below (Fig. 12.6).

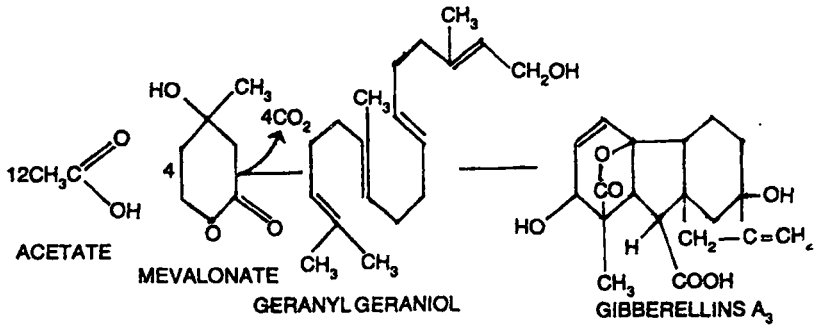

Fig. 12.6. Proposed scheme for the biosynthesis of gibberellic acid (GA_3).

Bioassay : The bioassay of the gibberellins is done by the barley endosperm test. The gibberellins promote seed germination and induce hydrolysis of starch. The barley endosperms are first of all incubated with the plant extract and release of reducing sugars is then measured. The response obtained may be compared with a standard experiment which is carried out with known concentrations of GA.

Functions : The various functions of gibberellins are as follows :

(a) When gibberellins are applied to intact plants, this results in increased stem and leaf extension growth.

(b) When aerial parts of genetically dwarf plants are sprayed with gibberellins, there occurs elongation of these plants.

(c) Gibberellins have been used successfully to get rid of seed dormancy in plants (*e.g.*, lettuce).

(d) Gibberellins have been especially useful to induce parthenocarpy in such fruits wherein auxins are unable to bring about this effect.

(e) As gibberellins delay senescence in plants, they may be used to stop abscission of flowers, fruits and leaves.

Action : Many effects of gibberellins have been found to be similar to those of auxins. Therefore, it was postulated that gibberellins act by changing the auxin level in plants. The observations supporting this postulation are increase in auxin level on applying GA, Prevention of GA affects by the use of antiauxins and replacement of GA requirement by auxin. In many

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other cases, it has been reported that auxins are not able to replace gibberellin effects. Thus, action of GA taking place through auxins may be applicable to some of its physiological effects. Induced synthesis of α-amylase by gibberellins takes place in barley endosperm. The induction gets inhibited by actinomycin D. It is most probably that gibberellins also act on several other physiological processes through enzyme induction.

Different forms of gibberellins have been synthesized from acetyl CoA and mevolonate sterols and terpenoids.

Gibberellins and Auxins : Cell extension which is mainly controlled by auxins, particularly IAA (Audus, 1955) has also been found to be controlled by one of the best known gibberellins—gibberellic acid; but the detailed study of the physiological properties of gibberellic acid and indole acetic acid reveals some interesting differences.

IAA induces the extension of etiolated coleoptiles and the growth response curve shows a linear elongation within the range of 0.01—1.00 μg/ml. GA however shows very little elongation of the coleoptile tip.

Auxins in addition to their effect on cell extension cause cell division—GA_3 however does not have any such effect on cell division. Thus treatment of cuttings with auxin helps in the initiation of roots, GA_3 antagonizes such root formation.

When auxins are applied on the decapitated tip of the main shoot, development of laterals is inhibited. GA_3 stimulates more rapid development of the laterals.

Auxins normally inhibit leaf abscission and produce abnormal cell proliferation in some tissues. GA_3 however, has no such effect.

All these differences between auxins and gibberellins lead naturally to assume that these two substances must act differently. But Brian *et al.* (1957) show that on producing their effect they must act similarly and so GA_3 depends for activity on the presence of IAA, although the effect of these two are complimentary.

12.3 Cytokinins

Carlos Miller and his associates in 1955, were successful in isolating a chemical factor for cell division from yeast DNA and called this *kinetin*. Later on this compound was found to be 6-furfuryl amino purine. Many other kinetins were also isolated in due course of time. They all induce cell division (cytokinesis). Therefore the name cytokinin is still used for all of them.

Root system is a major region of cytokinin synthesis.

Chemically the cytokinins have been found to be derivatives of purine bases. Structures of some natural cytokinins are given on page 283.

Bioassay : There are many types of bioassays which are used for cytokinins. However, the most rapid and sensitive one is the betacyanin synthesis test. There are certain species of *Amaranthus* which produce betacyanin in light but not in dark. Synthesis in dark has been promoted by cytokinins. This test has been found to be specific for cytokinins and a concentration of cytokinin as low as 30×10^{-7} M can respond to this test.

Functions : The various functions of cytokinins are as follows :

(a) As compared to gibberellins, cytokinins are more effective in delaying senescence in aerial parts of plants.

(b) Cytokinins have been employed successfully to induce flowering in short day plants.

(c) As compared to gibberellins, cytokinins have been found to be more effective in seed germination in dormant seeds.

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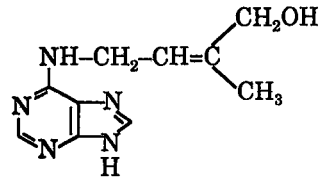
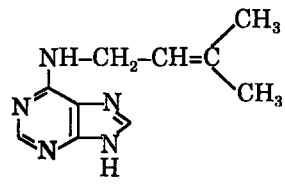
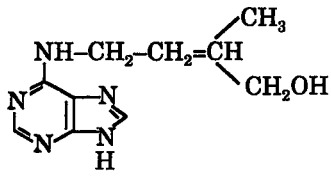


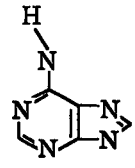
Zeatin (4-Hydroxy 3-Methyl but-2 enyl amino purine)



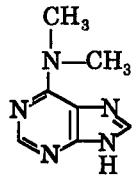
Isopentyl adenine



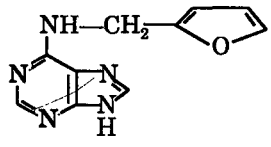
Dihydrozeatin



Methyl amino purine



Dimethyl aminopurine



Kinetin (6-furfuryl amino purine)

(d) Cytokinins are able to induce all divisions in cultures even if they are provided with concentrations as low as 10^{-8} ppm. The main role of cytokinins is to promote cytokinesis in cell division.

(e) The important role played by cytokinins is to mobilise solutes from one part of plants to the site of utilisation.

(f) Cytokinins cause changes in the protein and nucleic acid components of tissues which are the basis of the cytokinins effects on cell division (Guttaman; 1956) as well as on growth and mobilization actions. Cytokinins bring about the mobilization of various solutes including amino acids, auxin and phosphorus. They also delay the senescence of leaves particularly detached one when applied to the leaves. This is at least partly a result of their mobilizing effect. When leaves are detached there is extensive hydrolysis of proteins and RNA and translocation of amino acids, mineral salts and other solutes out of the leaves. Cytokinins apparently promote the synthesis of protein, RNA and other essential substances and also reducing translocation of such solutes out of the leaves because of their mobilizing influence.

Action : From the effects of cytokinins on DNA, RNA and protein synthesis it follows that they act through inducing gene expression. Synthesis of many enzymes has not been induced by cytokinins. They have been isolated in the t-RNA preparations obtained from several organisms but they could not be isolated in m-RNA preparations. Under experimental conditions it also has been reported that there occurs the incorporation of 6-benzyl amino purine (a cytokinin) into t-RNA of soyabean and tobacco callus. Therefore, it is more probably that the cytokinins affect the plant processes involving t-RNA.

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The origin and biosynthetic pathways for cytokinins are not fully understood. However, they are most probably synthesized in the roots. They are purine molecules and are therefore derived from normal nucleotide synthesis pathway. The isoprenoid chain of zeatin has been presumably synthesized from mevolonic acid.

The most important differences between the cytokinins and the other growth substances are that cytokinins are not acidic in reaction like auxins and gibberellins. It has been found by Wooley (1957), that plant hormones are more acidic because of the acidic nature of the plant cell walls; the alkaline materials are less mobile and cytokinins are the extreme case of non-mobility because of their non-acidic reaction.

12.4 Abscisic Acid

Abscission (*i.e.,* the falling of leaves, flowers and fruits) is a great practical problem in plants. Osborne (1955), Addicott (1963-65) and many co-workers have isolated several abscission-accelerating substances from cotton and many other plants. These substances have been named as *abscission I* and *II*. Later on Wearing and his associates however discovered dormancy-inducing substance from the leaves of *Acer (maple)* and they termed it as *dormin*. After determining the chemical nature of abscisin II and dormin, it was found that both these substances are identical. It was therefore decided in 1967 that abscisin II or dormin should be named *abscisic acid (ABA)*.

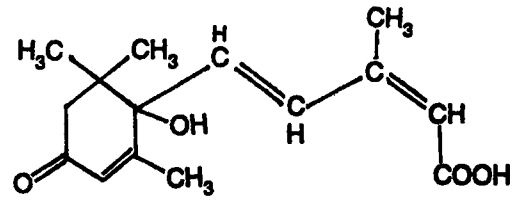

Fig. 12.7. Structure of abscisic acid (ABA).

Abscisic acid is a terpenoid and is unique among hormone in having an asymmetric carbon (the ring carbon to which the side chain is attached, Fig. 12.7), which means that two optical isomers are possible. The naturally occurring ABA is therefore represented as (+)–abscisic acid and the synthetic one as (–) abscisic acid.

Abscisic acid is a weak organic acid which is present in young buds or dormant seeds.

Abscisic acid inhibits rather than promoting growth. As it antagonises the effect of auxins and gibberellins, it inhibits phototropism, geotropism and cell elongation in different plant parts. Its role in controlling dormany has been established, besides its involvement in other development processes in plants such as senescence, abscission and flower initiation.

Abscisic acid is synthesized from mevolonic acid. However, the details of pathway are not known with certainty.

Biosynthesis of ABA : The biosynthesis of abscisic acid has still not been fully elucidated. One group suggests that ABA is synthesized via mevalonic acid, the pathway pattern followed for gibberellin biosynthesis. Since ABA is an antagonist of gibberellic acid, a switch over of the biosynthetic route from ABA to gibberellic acid or *vice versa* would alter the sequence of morphogenesis.

But because of their structural similarity with carotenoids, many however are of opinion that ABA arises by way of the breakdown of carotenoids. Zeaxanthin or violaxanthin is said to be the possible precursor of ABA (Addicott *et al.,* 1966). Taylor and Burden (1970) further suggested that the photo-oxidation or and biological oxidation of violaxanthin can give rise to *xanthoxin* which is ultimately converted to abscisic acid. According to Miborrow (1974) plastids especially the chloroplasts are the important sites of ABA biosynthesis.

Distribution of ABA : Abscisic acid is widely present in monocots, dicots and also in ferns. Fruits contained the highest ABA concentration.

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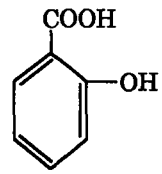
Physiological role of ABA : Majority of plant hormones promote particular process. Abscisic acid on the other hand is an inhibitor and acts contrary to the 'positive' hormones. The adjustment of the equilibrium between ABA and three other hormones determine whether a particular morphogenic process can occur or not. If ABA dominates, then the developmental process does not take place. If the 'positive' hormone gains the upper hand, then the development in question may occur. Thus the effect of IAA on *Avena* coleoptile curvature or coleoptile straight-growth is negated by ABA. With the increase of IAA conc. the ABA activity reversed. ABA also intract with gibberellins. The GA-induced synthesis of α-amylase and other hydrolytic enzymes in barley endosperm is inhibited by ABA. Like IAA the inhibitory effect of ABA on GA-induced enzyme synthesis is reversed by increasing conc. of GA. The growth of *Lemna* fronds is strongly inhibited by ABA. If ABA containing medium is added with cytokinin, the growth of the *Lemna* proceeds normally.

Another important role of ABA is in the stress physiology of plants. Under moisture stress condition, the leaves accumulate much ABA. Under the high concentration of ABA, the guard cells lose water and the stomata close. This aspects of ABA activity is being intensely studied nowadays.

Besides their usual role of lead and fruit abscission, bud dormancy and senescence of leaves, ABA inhibits genetic activity in inhibiting transcription and translation. All the other groups of plant hormones can promote the activity of certain genes. So the antagonistic effects would be intelligible on this molecular basis.

12.5 Phenolic Compounds

Besides abscisic acid there are many other phenolic compounds which have gained importance as plant growth regulators in recent years. The phenolic compounds have been found to occur in micro-organisms, pteridophytes gymnosperms and angiosperms. Earlier, most of the phenolic compounds are believed to be inhibitory in nature. From careful studies with the different concentrations of phenolic compounds it followed that many of them could stimulate physiological and biochemical processes at very low concentration. Salicylic acid is most well studied phenolic acid. It has been found to induce flowering in a variety of plants including some crop plants at low levels. It has been also found to increase the growth of daughter fronds of *lemna minor*, bud formation in cultured tobacco cells and nitrate assimilation and related enzymic activities in maize seedlings.



Salicylic acid (OH benzoic acid)

There are several other phenolic acids such as caffeic chlorogenic, gallic cinnamic acid, etc. which also have growth regulating properties. It is not known with certainty that salicylic and other phenolics act directly on the biochemical processes or through some other growth regulators such as auxin.

12.6 Ethylene

The story of ethylene began as early as 75 years ago when the effect of ethylene on the tropistic response of roots was observed. This synthetic gas (obtained by incomplete combustion of carbon-rich compounds like coal, petroleum etc.) found to have caused leaf abscission, abnormal curling of leaf blade and petiole, flower petal discolouration, inhibition of stem and root elongation. But

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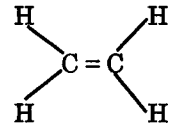
Gane (1934) first observed that ethylene is actual a natural product of ripening fruits. Denny and Miller (1935) further exploited this idea and observed that ethylene is produced not only in ripering fruits but also by flowers, seeds, leaves and even by roots. Further their presence in plant tissues brings about regulatory effect led to consider that ethylene is a plant hormone. Normally a small amount of ethylene (less than 0.1 ppm) is found to be present in plant tissue, although a high concentration may be present in the flowers of *Vanda* (orchid).

The rate of ethylene production is very susceptible to environmental factors. According to Burn and Thimann (1959) high or low temperature as well as low oxygen level depress the ethylene production. These effects are usually exploited in fruit-storage techniques where limitation of ethylene production can improve the storage life of fruits. Red light also causes a sharp depression of ethylene in plant tissue. Another important influence of ethylene production is stress or injury. In fruits, the stimulation of ethylene production by cuts or bruises is very great and bear considerably on the effectiveness of storage of the fruits. Robitaille (1973) however observed that in apple there is no stimulation of ethylene production by wounding.

Ethylene is a gaseous hormone and since it is highly soluble in water and also in lipophilic system it can easily move through plant tissues. Their movement through tissues may be through air spaces and hence related to tissue porosity, its lipophilic quality permits ready movement through membrane also.



Or $H_2C = CH_2$

Structure of ethylene

Biosynthesis of ethylene : Though the structure of ethylene is very simple and produced in a number of plant organs the actual biosynthetic steps are very uncertain. According to Liebermann *et al.* (1966) methionine is said to be an early precursor of ethylene formation. The enzyme responsible for catalyzing methionine to ethylene *in vitro* have been isolated from plants. Some of the intermediate steps in the ethylene biosynthesis is given below :

Methionine ⟶ α-keto γ-methyl mercaptobutyrate (KMB) ⟶ Methional ⟶ Ethylene

Another possible substrate for ethylene biosynthesis is fatty acids. Linolenic acid, an 18-carbon unsaturated fatty acid may serve as a precursor of ethylene. There is a close correlation between ethylene formation and fatty acid metabolism. Since lipids are components of cell membrane, fatty acids might be released due to wounding or tissue damage accompanied by the release of enzymes capable for converting fatty acids to ehylene. This fact however is of interest as it has been known that wounding or disruption of cell frequently leads to ethylene formation.

Of other possibilities ethylene production was also demonstrated from sugar and acetate. The way through which this simple compound is synthesized in plants is difficult to understand at present.

Physiological Role of Ethylene

The various functions of ethylene as an hormone are as follows :

(*i*) It is useful in breaking dormancy and sex expression studies. For example, ethylene treatment causes the formation of female as opposed to male flowers. Thus, it has been found to be antiauxin and antigibberellins.

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(*ii*) As ethylene increases chlorophyll loss and softening of fruits, it is involved in the ripening of fruits.

(*iii*) It has been found to increase respiration by increasing glycolytic and TCA cycle activity.

(*iv*) It has been found to inhibit hook opening in lettuce, pea and bean seedlings and growth of hypocotyl in many plants.

(*v*) It is participating in prototropic movement of mung bean stem probably by acting as an inhibitor of auxin transport.

(*vi*) In some plants, ethylene has been found to induce synthesis of biologically active compounds.

(*vii*) It has been found to induce the phenyl ammonia lyase which is key enzyme in the synthesis of flavonoids.

(*viii*) It has also been found to induce the synthesis of enzyme kianse in bean leaves and β-1, 3-glucanase in tomato and bean leaves.

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13 Plant and Animal Pigments

13.1 Introduction

The natural pigments are intensely coloured substances which occur in the plants as well as in animals. When those occur in plants, these are known as plant pigments, and when these occur in animals, these are known as animal pigments. However, some pigments are known which are found to occur in both plants and animals. For example, carotenoids in plants occur as colloidal suspensions of their esters or as protein complex while these in animals occur in solution in fats or in combination with proteins.

Pigments not only impart a characteristic colour to the particular tissue or organ where they exist, they are also involved in trapping light for photosynthesis and several other physiological processes.

Plant pigments are the synthetic products of the plants, formed during the metabolic activities of the cell. Various pigments are found in plants, of which *chlorophylls* are the most important. They represent the green colour of the plants and are always associated with the plastids. Their main role is in the photosynthetic process of plants. Besides, there are the yellowish coloured *carotenoids* which always remain in association with chlorophylls or may be independently dissolved in the plastids. They are never found in the cell sap of the plant. *Anthocyanins* are another group of colouring matters which are always dissolved in the cell sap. They are responsible mainly for the bright colour of the flowers and fruits. Their main role is to attract the insects for pollination and dispersal of seeds.

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13.2 Plant Pigments

Some important plant pigments are described as follows :

1. Chlorophyll

Introduction : It is the green colouring matter of leaves and green stems. This substance is essential for carrying out the photosynthesis. The latter is very important process which synthesises carbohydrates, fats and proteins by absorbing light energy in the presence of chlorophyll.

In 1818, Pelletier and Caventor named the green pigment in leaves as chlorophyll. In 1864, Stokes attempted to show by spectroscopic evidence that chlorophyll is not one compound but a mixture of two or more compounds. However, his work was ignored for a number of years until Willstatter (1911) made a thorough study of chlorophyll and confirmed the view point of Stoke. According to Willstatter, chlorophyll obtained from a wide variety of sources was a mixture of two compounds called chlorophyll-*a* and chlorophyll-*b*.

When a light petrol solution of chlorophyll is shaken with aqueous methanol, chlorophyll-*a* remains in the light petrol whereas chlorophyll-*b* goes into the aqueous methanol. In higher plants, chlorophyll-*a* and chlorophyll-*b* occur in proportions of approximately 3 : 1 in natural chlorophyll.

From natural chlorophyll, the two compounds can be separated by column chromatography using alumina, sugar, starch, etc.

Chlorophyll-*a* is a bluish-black solid whereas chlorophyll-*b* is a dark green solid. However, both chlorophylls give a green solution in organic solvents.

The molecular formula of chlorophyll-*a* is $C_{55}H_{12}N_4O_5Mg$ while the molecular formula of chlorophyll-*b* is $C_{55}H_{70}N_4O_6Mg$.

Chlorophyll-*a* exhibits characteristic maxima at 380, 418, 428, 510, 580 and 700 nm whereas chlorophyll-*b* exhibits characteristic maxima at 428,464 and 675 nm. These characteristic absorption maxima have been employed to estimate the amounts of each chlorophyll in a mixture (Fig. 13.1).

Green algae and higher plants contain two types of chlorophylls, Chl *a* and Chl *b*. Both are soluble in organic solvents. Chl *a* is present in all photosynthetic organisms in which there is evolution of oxygen during photosynthesis. The following types of Chl *a* are known :

Chl *a* 660, Chl *a* 670, Chl *a* 680, Chl *a* 685, Chl *a* 690 and Chl *a* 700-720. The short wavelength forms of Chl *a* are mainly present in photosystem II, while the long wavelength forms are mainly present in photosystem I.

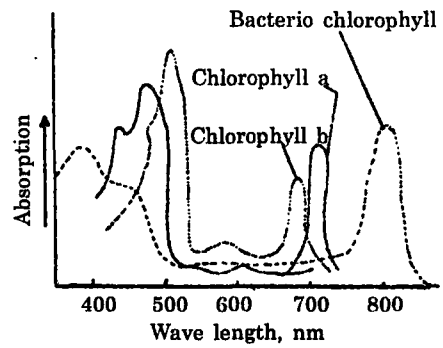

Fig. 13.1. The absorption spectra of chlorophylls.

Chl *b* is present in all green algae and higher plants. Most of the Chl *b* is present in PSII. There are two forms of Chl *b*, CHl *b* 460 and Chl *b* 650.

Brown algae contain a compound called Chl *c* which is related to chlorophyll. Chl *d* has been reported in the red algae.

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Structure of chlorophyll : The empirical formula of chlorophyll *a* is $C_{55}H_{72}O_5N_4Mg$. Chlorophyll *a* is a blue-green microcrystalline solid, consisting of a 'head' and a 'tail.' The head consists of a *prophyrin ring* or *tetrapyrrole nucleus*, from which extends a tail made up of a 20-carbon grouping called the *phytol*. The *porphyrins* are complex carbon-nitrogen molecules that usually surround a metal. In *chlorophyll* the porphyrin surrounds a *magnesium* ion while in *hemoglobin* it surrounds an *iron* ion. The cytochromes of the electron transport system also have porphyrin rings. The basic unit of the porphyrin ring is the *porphobilinogen* molecule. Four such *pyrroles* make up units of the *tetrapyrrole* structure. *Phytol* is a long straight chain alcohol containing a double bond. It may be regarded as a hydrogenated carotene (vitamin A). Its formula is $C_{20}H_{39}$.

Chlorophyll-*b* has the empirical formula $C_{55}H_{70}O_6N_4Mg$. It is a green-black microcrystalline solid. It differs from chlorophyll *a* in having an *aldehyde* (CHO) group attached to carbon atom 3, instead of a *methyl* (CH_3) group.

Bacteriochlorophylls : The photosynthetic pigments of the purple and green bacteria are bacteriochlorophylls *a, b, c, d* or *e* and a variety of carotenoids, the characteristics of the different bacteriochlorophylls are given in the Table 13.1.

Table 13.1
TYPES OF BACTERIA CHLOROPLAST

Designation of Jenson et.al. 1964	*Former designation*	*Characteristic absorption Maxima in living cells (nm)*
BChl *a*	Bacteriochlorophyll	375, 590, 805, 830-890
BChl *b*	Bacteriochlorophyll b	400, 605, 835-850, 1020-1040
BChl *c*	Chlorobium chl 660	Long wavelength absorption max 745-755
BChl *d*	Chlorobium chl 650	-do- 705-740

Biosynthesis of chlorophylls : Until 1960 very little was known about how an organism synthesized these complex chlorophyll molecules inside the chloroplasts (except blue-green algae which have no chloroplasts).

In 1961, Gracing and Mauzerall proposed a scheme (Fig. 13.3) for biosynthesis of chlorophyll-*a*. With the exception of one or two steps, majority of the stages have been clearly investigated. According to Shemin (1956) the early precursors of porphyrin synthesis are glycine and succinyl Co-A, which are considered to be parts of the TCA cycle. These then by decarboxylation form δ-aminolaevulinic and (ALA) *via* α-amino β-keto adipic acid through a cycle known as *Shemin cycle.*

$$HOOC{-}CH_2{-}CH_2COS.Co\text{-}A + H_2C\begin{matrix}NH_2\\COOH\end{matrix} \longrightarrow HOOC{-}CH_2{-}CH_2{-}CO{-}CH_2{-}NH_2 + CO_2$$

(Succinyl Co-A) (glycine) (δ-amino laevulinic acid)

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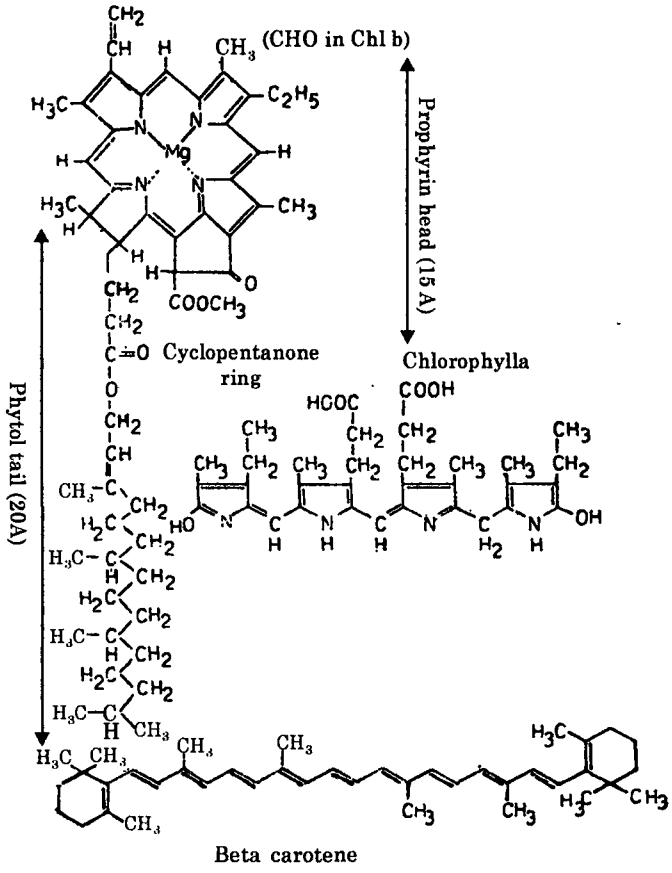
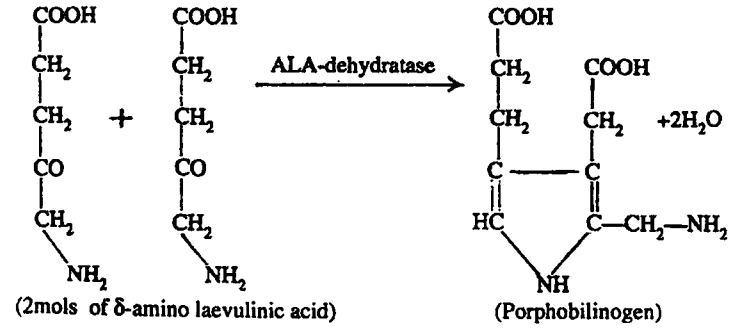


Fig. 13.2. Photosynthesis pigments : Cholorophyll *a* and *b*, phycocyanin and β-carotene.

Two molecules of δ-amino laevulinic acid units head to tail to form the pyrrole derivatives—porphobilinogen (PBG).



Four molecules of porphobilinogen (PBG) then give rise to uroporphyrinogen III with the enzyme *porphobilinogen deaminase* (it is also known as *urogen I synthetase* and *urogen* 3-*co-synthetase*).

4 mols. of Porphobilinogen ⟶ Uroporphyrinogen III + $4HH_3$

Uroporphyrinogen III then by decarboxylation with the enzyme *urogen decarboxylase* yields a molecule with four methyl substituents called coproporphyrinogen III. Coproporphyrinogen III then forms protoporphyrinogen which ultimately oxidises to protoporphyrin IX which is catalysed by the enzyme *coproporphyrinogen oxidative decarboxylase.*

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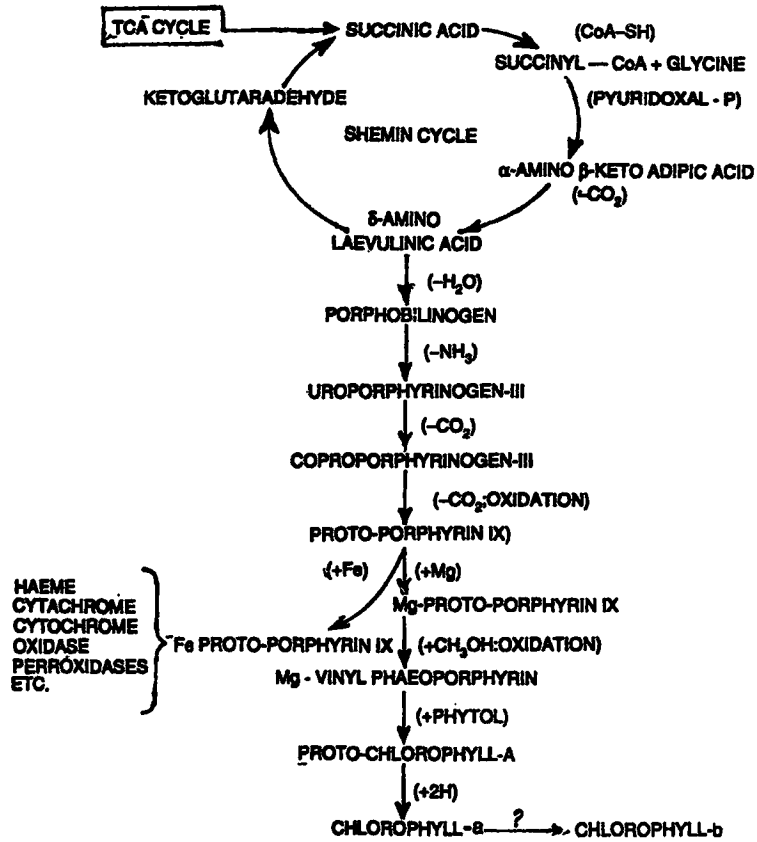

Fig. 13.3. Biosynthesis of chlorophyll-α.

$$\text{Coproporphyrinogen III} \xrightarrow[-4H]{-2CO_2} \text{Protorphyrinogen} \longrightarrow \text{Protoporphyrin IX}$$

Protoporphyrin IX is the immediate common precursor of chlorophyll and other porphyrins (protohaeme); interaction of Mg in the centre of this molecule produces Mg-protoporphyrin. Mg-protoporphyrin then through a series of interconversions produces chlorophyll. We known very few of the enzymatic steps involved in this transformation and the intermediates.

Introduction of Fe^{++} to protoporphyrin IX produces haeme, cytochorme oxidase, peroxidases and catalases.

Mg-protoporphyrin monomethyl ester then converts to Mg-vinyl pheoporphyrin a_5 (protochlorophyll). The reduction of this compound to chlorophyll involves reduction of the IVth tetrapyrrole ring and an esterification of this residue by phytol, a long chain alcohol. The intermediates of these reactions probably never occur in the free state. It should be mentioned that the reactions take place in higher plants and in some algae only in the presence of light.

Chlorophyll-*b* does not originate from chlorophylla-*a* or by the same process through which chlorophyll-*a* is synthesized. The details are however not known.

2. Carotenoids

Introduction : Carotenoids are fat soluble yellow or red pigments which are considered to be polyenes. These are composed of isoprene units, usually eight, arranged in such a way that in

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the middle of the molecule, two methyl groups are present in 1, 6-positions to each other while in all other side chin methyl groups are present in 1, 5-positions. In most of the carotenoids, their central portion contains *a long conjugated chain* which is composed of four isoprene units, the centre two of which are linked tail to tail. At the two ends of chain, there may be two open chain structures, or one open-chain structure and one ring or two rings. *The colour of the carotenoids has been attributed to the extended conjugation of the central chain.*

As the carbon skeleton of carotenoids has a polystyrene structure, they may be considered to be *tetraterpenes.*

From X-ray analysis of majority of natural carotenoids, it follows that the doubles bonds are in all- *trans*—position. In a few natural carotenoids, double bonds are in *cis*-trans-position. All *trans*-carotenes may be written as follows :

where R may be an open chain structure or a ring system.

In plants, chlorophyll occurs in combination with carotenoids, carotene and lutein. In photosynthesis, the carotenoids act as photosensitisers in conjunction with chlorophyll. When chlorophyll is absent as in fungi, then the carotenoids are considered to be responsible for colour.

There are two types of carotenoids, *carotenes* and *carotenols* or *xanthophylls. Carotenes, e.g.,* β-carotene, are hydrocarbons. Most of the carotenes are present in photosystem II.

In nature β-carotene occurs in all the green plants. It often occurs associated with chlorophyll (Fig. 13.4). Carrots are considered to be the richest sources of β-carotene.

The structure of β-carotene is shown in Fig. 13.4. The β-carotene is the most abundant carotenoid. It is yellow orange in colour and its absorption maxima is 430-480 nm.

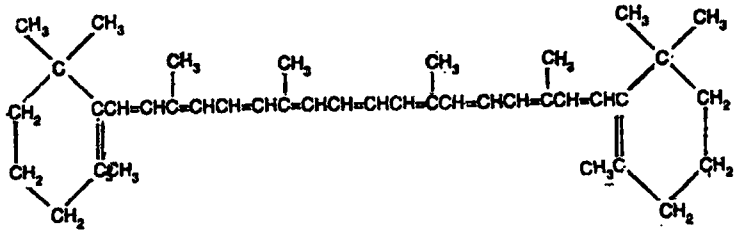

Fig. 13.4. Structure of β-carotene. Note the symmetry and two ionone rings at the two ends.

Carotene is the most common carotenoid found in plants. It possesses an orange-yellow colouration. The name "carotene" as first given in 1831 by Mackenroder to a substance isolate from carrots. The structure of the carotene was definitely established only after 1925 from the work of Karrer, Kuhn. Lederer. Carotene is the derivative of the red pigment—*lycopene,* found in tomato and other fruits and flowers. It is a highly unsaturated straight chain hydrocarbon having the formula $C_{40}H_{56}$.

Four important naturally occurring carotenes with the same chemical composition ($C_{40}H_{56}$) are named α-carotene, β-carotene, γ-carotene and δ-carotene of which β-carotene is only symmetrical and other three carotenes are asymmetrical. In all types of carotenes, there are *ionone* rings either at one or both ends of the molecule. α-and β-carotene contain two ionone rings and γ-and δ-carotenes have only one such ring. The most characteristic feature of these carotenes is the presence of a ring at one or both hydrocarbon chains (Fig. 13.4).

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Like chlorophylls, carotenes are insoluble in water but readily soluble in alcohol, chloroform, benzene etc. They show the property of fluorescence—orange-red by transmitted light and green-blue by reflected light. Being unsaturated they are quickly oxidised in air changing till original colour takes place in scraped carrot. Another property of β-carotene is that it can absorb the blue-violet portion (449mμ-478mμ) the visible spectrum. Xanthophyll however absorbs light of 440mμ and 490mμ. The β-carotene can be converted to vitamin A_1 *in-vivo* and so it is termed as *provitamin* A_1.

The oxygenated carotenoid compounds are known as *xanthophylls*. These are yellow or brown pigments having the formula $C_{40}H_{56}O_2$. The oxygen atom is attached to the terminal ring of carotene. The most important xanthophyll is *lutein* obtained from the green leaves, flowers and fruits of sunflower. Lutein is an oxygenated derivative of α-carotene. Other xanthophylls are *lycoxanthin* obtained from tomato, *zeaxanthin* obtained from yellow corn, *cryptoxanthin* from green leaves and yellow corn, *violaxanthin* from green algae and *fucoxanthin* from brown algae.

Table 13.2
MAJOR XANTHOPHYLL PIGMENTS OF GREEN LEAVES (FROM GOODWIN, 1960)

Pigment	*Structure*
Lutein	3, 3-Dihydroxy α-carotene
Cryptoxanthin	3-Hydroxy β-carotene
Zeaxanthin	3, 3-Dihydroxy β-carotene
Violaxanthin	5, 6, 5′, 6′-Diepoxyzeaxanthin
Neoxanthin	$C_{40}H_{56}O_4$ (?)

A few naturally occurring carotenoids contain carboxyl group which are found to be a derivative of other carotenoids due to oxidative cleavage. The important example is *bixin* ($C_{25}H_{20}O_4$) obtained from the pods of *Bixa orellane, crocetin* ($C_{20}H_{24}O_4$) obtained from *Crocus sativus* (saffron) etc.

Xanthophylls or carotenols are naturally occurring carotenoids which contain an oxygen function. These are also known as phytoxanthins. Most of xanthophylls possess the same carbon skeleton as carotenes or lycopene except flavoxanthin. Most of xanthophylls have been synthesised. Common examples of xanthophylls are xanthophyll (lutein), zeaxanthin, and spirolloxanthin.

The structure of spirolloxanthin (an important xanthophyll) is shown in Fig. 13.5.

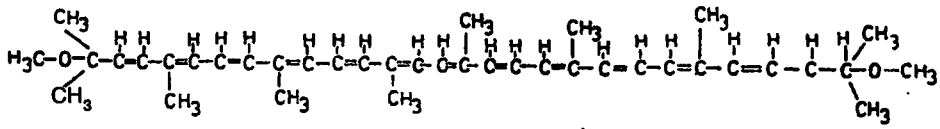

Fig. 13.5. Structure of spirolloxanthin (a xanthophyll).

Biosynthesis of carotenoids : Although the biosynthetic process in the formation of carotenoids has been known considerably, very little is known about the metabolic interrelations among the various groups of carotenoids. It has been suggested that the C_{40}-compound which is formed first, is a colourless highly saturated *phytoene* (*e.g.*, tetrahydrophytoene) which by subsequent dehydrogenation gives rise to coloured carotenoids (*e.g.*, lycopene) (Bonner, 1946; Griffiths and Stanier, 1956). According to Mackinney (1956), however, the biosynthesis of carotenoids does not agree with this hypothesis and according to him colourless phytoene and coloured carotenoids are synthesized by separate pathways from a common precursor and each C_{40}-compound is formed independently.

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The biosynthesis of β-carotene (Grob and Butler, 1956) involves the initial conversion of C_2 units from acetic acid (probably acetyl Co-A). The first step of carotenoid synthesis involves the formation of three acetyl Co-A. Then by two successive reactions from acetoacetyl Co-A and then to hydroxy methylglutaryl Co-A. Hydroxy methylglutary Co-A then reduced to mevalonic acid in presence of $NADPH+H^+$. Mevalonic acid is then phosphorylated in presence of ATP molecules to from mevalonic acid pyrophosphate. Then decarboxylation of mevalonic acid pyrophosphate with ATP and decarboxylating enzyme yields isopentenyl pyrophosphate (IPP)—a five carbon isoprenoid unit and from which all other carotenoids are derived.

Light is not essential in the formation of carotenoids. They are usually formed in darkness.

Functions of carotenoids in plants : The exact function of carotenoids in plants is not known with certainly and this still requires further investigation. However, different views given by various workers as follows :

1. According to *Noack*, carotenoids act as light filters in plants which protect chlorophyll from destruction with strong light.
2. According to *Went*, the main function of carotenoids is to protect sensitive enzymes in the cell.
3. According to *Warburg*, the main function of carotene and xanthophyll is to help in assimilation.
4. Some carotenoids participate in the reproduction of algae.
5. Carotenoids also play role in some redox reactions which are taking place in plants. For example, the colloidal solutions of carotenes act as hydrogen acceptor.
6. According to Robinwith, carotenoids have no primary role in the process of photosynthesis. However, they act as photosensitizers in combination with chlorophyll. When a plant is exposed to light, the carotenoids take energy from light protons and get excited and then they transfer excitation energy to chlorophyll.

$$\underset{\text{Light Photon}}{\text{Carotenoid} + h\nu} \longrightarrow \underset{\text{(Excited)}}{\text{Carotenoid}^*}$$

$$\underset{\text{(Excited)}}{\text{Carotenoid}^*} + \text{Chlorophyll} \longrightarrow \text{Chlorophyll}^* + \underset{\text{(Excited)}}{\text{Carotenoid}}$$

Functions of carotenoids in animals : In animals, the carotenoids play the following important roles :

(a) In the intestinal tract or in liver, most of the carotenoids are converted into vitamin A and thus, they act as provitamins. For example, β-carotene on absorbing two molecules of water is converted into two molecules of vitamin A.

In γ-carotene, there is only one β-ionone ring and, therefore, it is, converted into only one molecule of vitamin A and hence it is less biologically active than β-carotene. The conversion of β-carotene and carotene in the intestinal tract or in liver takes place in the presence of enzyme called *carotenase*. However, the mechanism of conversion of β-carotene into vitamin A is not understood completely but it has been established experimentally by T. Moore.

Moore took some rats whose livers did not contain vitamin A. When these rats were given a diet which did not contain vitamin A but contained β-carotene, their livers accumulated vitamin A. This experiment revealed that β-carotene is converted into vitamin A.

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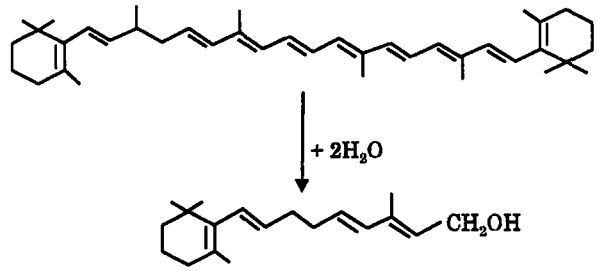

Vitamin A (retinol)

The conversion of β-carotene into vitamin A has been successfully carried out by chemical methods, *i.e.*, by the oxidation of β-carotene with hydrogen peroxide in glacial acetic acid.

$$\beta\text{-Carotene} \xrightarrow{H_2O_2/CH_3COOH} \text{Retinal} \longrightarrow \text{Retinol (Vitamin A)}$$

When animals are deficient in vitamin A, then 70-80% of carotene gets converted into vitamin A. But, if the animals are not deficient in vitamin A, only small percentage of carotene gets converted into vitamin A.

The form in which carotenes are given to animals also influences their conversion and absorption. For example, β-carotenoids dissolved in animal or vegetable fats undergo easy conversion as well as easy absorption than the carotenoids dissolved in paraffin oil or ethyl-oleate.

(b) In the process of vision, some carotenoids also play an important role by acting as light filters which only allow rays of a certain wavelengths to reach the innermost part of the eye and then these affect the photo labile cells of that part of eye.

A carotenoid will be converted into vitamin A provided it contains an unsaturated β-ionone nucleus and an unsaturated side-chain; both these are also present in vitamin A. Due to this reason, α-carotenone and β-eninone are not active biologically due to the absence of β-ionone of the former and side-chain in the latter compound.

3. Phycobilins

Phycobilins are water-soluble open chain tetrapyrroles which are present in red algae and blue-green bacteria (Cyanobacteria). There are two kinds of phycobilins, *phycocyanins* and *phycoerythrins*. Phycocyanins are predominate in the blue-green bacteria, while phycoerythrins predominate in the red algae-. Phycobilins are mainly present in PSII, but are also present in PSI.

Phycobilins also absorb light. However, the light absorbed by these pigments gets transferred as excitation energy to chlorophylls where it is used to carry out photosynthesis. Therefore, the main function of phycobilins is to help chlorophylls in the trapping of light energy and they are termed as 'accessory pigments.'

The two important phycobilins are—phycoerythrobilin (a red phycobilin) and phycocyanobilin (a blue phycobilin). They constitute the most important pigments of red and blue green algae, they are found in

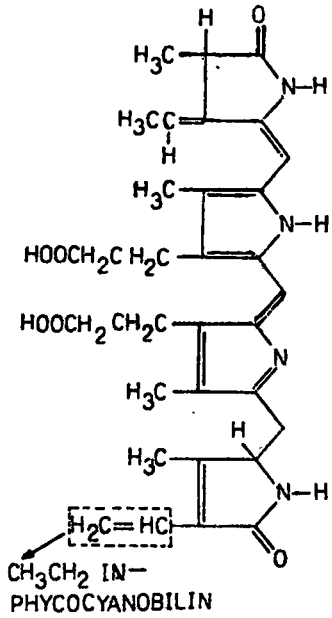

Fig. 13.6. Structure of phycoerythrobilin.

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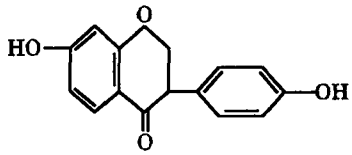
the cell as protein conjugates. The phycobilins are linear tetra-pyrroles and unlike chlorophylls they lack Mg^{2+} (Fig. 13.6).

4. Flavonoids

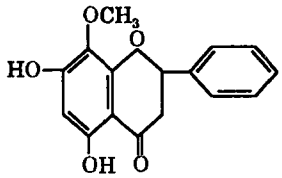
The flavonoids impart mostly red, blue and violet colour to the plant organs. Chemically, the flovonoids have been found to be phenolic compounds and most of them are having a $C_6—C_3—C_6$ skeleton. The structures of some important coloured flavonoids are given as follows :

(*i*) *Diadzein*



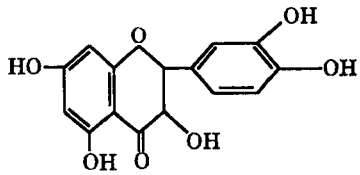
colourless or pale yellow

(*ii*) *Wogonin*



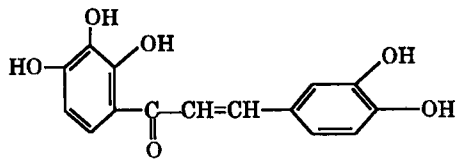
light yellow

(*iii*) *Quercetin*



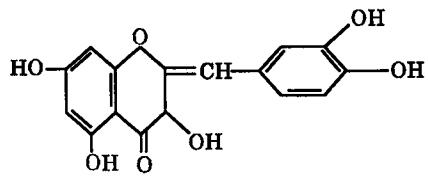
yellow

(*iv*) *Okanin*



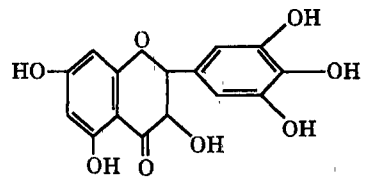
yellow to orange

(*v*) *Aureusidin*



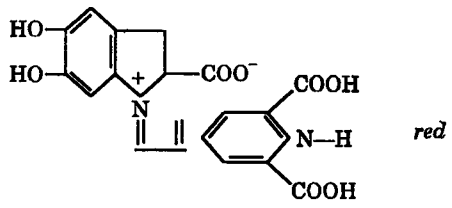
yellow to orange red

(*vi*) *Anthocyanins (Delphidin cation)*



red

(*vii*) *Betacyanins (Betanidin)*



red

The distribution of flavonoids in plant kingdom is more or less of taxonomic significance. Algae, fungi and bacteria lack any kind of flavonoid, whereas mosses have a few types of them. Ferns and, gymnosperms have many types of simple flavonoids whereas angiosperms have a whole range of flavonoids. Highly complex forms of flavonoids occur in the highly evolved families like compositae.

Biosynthesis : The precursors of flavonoid biosynthesis include shikimic acid, phenylalanine, cinnamic acid and *p-coumaric* acid. Shikimic acid acts as an intermediate in the biosynthesis of aromatic amino acids. The pathways for the biosynthesis of flavonoids may be summarised as below :

(*i*) Shikimic acid + Phosphoenol pyruvic acid ⟶ Prephenic acid

(*ii*) Prephenic acid ⟶ *p*-Hydroxy phenyl pyruvic acid

(*iii*) *p*-Hydroxy phenyl pyruvic acid ⟶ Tyrosine

(*iv*) Tyrosine ⟶ *p*-Coumaric acid

(*v*) *p*-Coumaric acid ⟶ Chalones

(*vi*) Chalones ⟶ Different flavonoids

5. Biloproteins (Phycobilins)

These are the main group of accessory plant pigments found only in certain algae. These red and blue pigments, *phycoerythrins* and *phycocyanins* were collectively called previously as *phycobilins*. The name 'phycobilins' was first proposed by Lemberg (1928) as this pigment has got a chemical affinity with the bile pigments of the animals. But O' heocha (1962) observed that the free pigment cannot be separated from a protein moiety and the name of the pigments was therefore changed from phycobilins to biloproteins to indicate the existence of the pigment-protein complex.

Biloproteins are present in only 3 algal divisions—the Cyanophyta, Rhodophyta and Cryptophyta, The species of *Cryptophyta-Cryptomonas, Rhodomones* etc. however contain some unknown phycocyanin and phycoerythrin. These are the main photosynthetic pigments of these algal groups. These pigments are associated with the protein, so the study of these pigments comes from the studies of the pigments-protein complex. Their presence in higher plants and green algae is still uncertain. Biloproteins differ from the other important plant pigments like chlorophylls and carotenoids in that these are water soluble, further these are associated with the plastids of red algae and with the cytoplasmic lamellae of the blue-green algae. In higher plants chlorophylls and carotenoids may be associated with proteins in the grana of the plastids like biloproteins, but their connection is very unstable. Whereas in biloproteins this protein-pigments association is very stable.

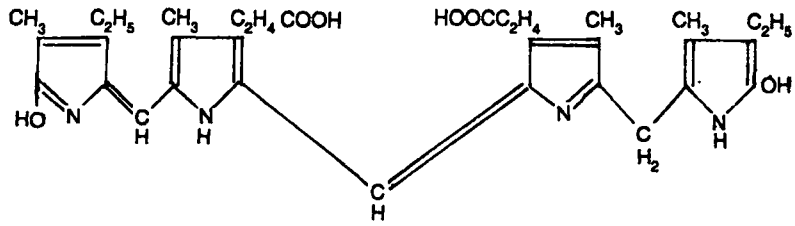


Fig. 13.7. The structure of the prosthetic group of phycocyanin. Phycoerythrin is similar in general structure, but here the prosthetic groups are attached to proteins.

Regarding the chemical structures of these compounds, it had been found that these consist of an open chain tetrapyrrole. They are the compounds of carbon, hydrogen, oxygen and nitrogen and with no metal or phytol in their chemical structures. The chemical formula of phycocyanin is $C_{34}H_{44}O_8N_4$. Phycoerythrin, however, contains two extra hydrogens and the formula is $C_{34}H_{46}O_8N_4$. The molecular weights of these pigments are 273,000 and 290,000 respectively. Although different forms of phycoerythrins and phycocynins have been isolated from different plants, they are basically same; only difference is in their nature of protein components rather than the pigments itself. As for example, the forms R-phycoerythrin and C-phycoerythrin differ on the plant source and consequently on the nature of protein part. The former has been isolated from red algae, whereas the latter from blue-green algae. Similar is the case of R-phycocyanin and C-phycocyanin. When they are isolated both from red and blue-green algae they are known as P-phycocyanin and P-phycoerythrin respectively.

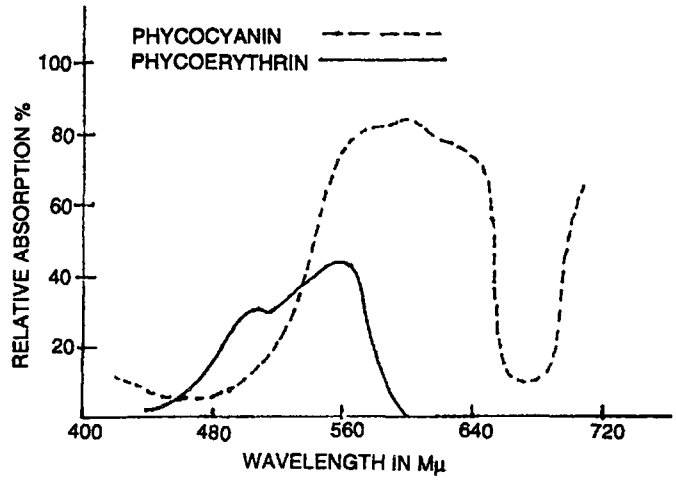


Fig. 13.8. Absorption spectra of phycocyanin and phycoerythrin.

Absorption spectra of biloproteins clearly indicate that these pigments are active in the transfer of light energy to chlorophyll for their uitlization in photosynthesis. R-phycoerythrin (mainly obtained from red algae) had three absorption zones at 495, 540 and 565 mμ and the maximum absorption being in the green region (Fig. 13.8). C-phycoerythrin (mainly obtained from blue-green algae) has got a maximum absorption in the green region (550 mμ). Another phycoerythrin, P-phycoerythrin has got an absorption spectra at green (550 mμ) and yellow (565 mμ) regions. For R- and C-phycocyanin the maximum absorption peak is at orange (615 mμ), a small absorption band has, however, been found in the green (550 mμ) in case of R-phycocyanin. In case of P-phycocyanin the absorption peaks move further to orange (650 mμ).

Like carotenoids, the role of biloproteins in photosynthesis is indirect. The energy absorbed by these pigments transmit their energy to chlorophyll, when they become 'active' in photosynthesis. The photosynthetic efficiency of these pigments is far greater than chlorophyll-*a*. The role of chlorophyll-*a* in blue-green and red algae is minor and subsidiary. Major light absorption takes place through these biloproteins.

Table 13.3
PIGMENTS IN PLANTS AND PHOTOSYNTHETIC BACTERIA

Group of Plants	*Chlorophylls*	*Carotenoids*	*Other Pigments*
Green plants	*a* and *b*	β-Carotene and lutein	Anthocyanins (non-photosynthetic)
Green sulphur bacteria	Bacterioviridin	Several	—
Purple sulphur bacteria	Bacteriochlorophyll	Spiriloxanthin	—
Diatoms	*a* and *c*	Fucoxanthin	—
Brown algae	*a* and *c*	Fucoxanthin	—
Yellow-green algae	*a* and *e*	Several	—
Red algae	*a* and *d*	β-Carotene and lutein	Biloproteins (phycoerythrin and phycocyanin)
Blue-green algae	*a*	Several	Biloproteins

ANIMAL PIGMENTS

1. Bile Pigments

Human bile secreted by the liver is having golden or brownish yellow colour which is due to the presence of two pigments *bilirubin* and *biliverdin*. These two pigments are termed as bile pigments. These two pigments are interconvertible by involving oxidation-reduction process. Biliverdin is green in colour and is a tetrapyrrole compound, which does not have a metallic atom (Fig. 13.9). The oxidation of this pigment yields blue green pigment whereas reduction produces biliverdin having red colour.

$$\underset{\text{(green)}}{\text{Biliverdin}} \xrightarrow{+H_2} \underset{\text{(red)}}{\text{Bilirubin}} \xrightarrow{+H_2} \underset{\text{(colourless)}}{\text{Urobilinogen}} \xrightarrow{-H_2} \underset{\text{(brown)}}{\text{Urobilin}}$$

$$\downarrow -H_2$$

Blue green pigments

The bile pigments are obtained from the haeme of hemoglobin from aged red blood cells and other haeme proteins like myoglobin, catalase, cytochromes, peroxidase etc. The bile pigments on degradation further by the micro-organisms which are present in the intestine yield brown pigment, called stercobilin, the chief pigment of faeces.

Haeme ⟶ protoporphyrin IX ⟶ bilirubin and biliverdin ⟶ stercobilin

2. Haem

Introduction : Haemoglobin is a component of red blood cells and is responsible for carrying oxygen from the lungs through the arteries, arterioles and capillaries to the tissues. Furthermore, it assists in carrying carbon dioxide from the tissues to the lungs.

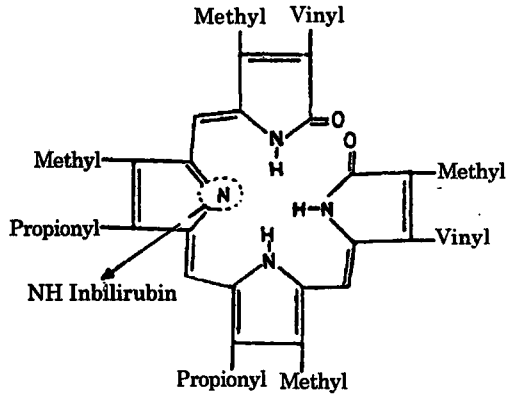


Fig. 13.9. Structure of Biliverdin.

Haemoglobin occurs in all vertebrates (few exceptions are known) and in many invertebrates. The haemoglobin consists of two parts : *(a)* globin and *(b)* haem. The globin is a protein part and its percentage in hemoglobin is 94%. Haem is a prosthetic part (non-protein part) and its percentage in haemoglobin is 6%.

Structure : Haem is an iron-protoporphyrin complex which is of two types :

(a) When the iron atom present in the haem is in the ferrous state, it is known as ferrous protoporphyrin, ferroprotoporphyrin, protohaem or *haem*. This molecule is electrically neutral.

(b) When the iron atom present in the haem is in the ferric state, it is known as ferric protoporphyrin, ferriprotoporphyrin or haemin. This molecule carries a unit +ve charge. Haemin can be prepared by warming blood with acetic acid and sodium chloride.

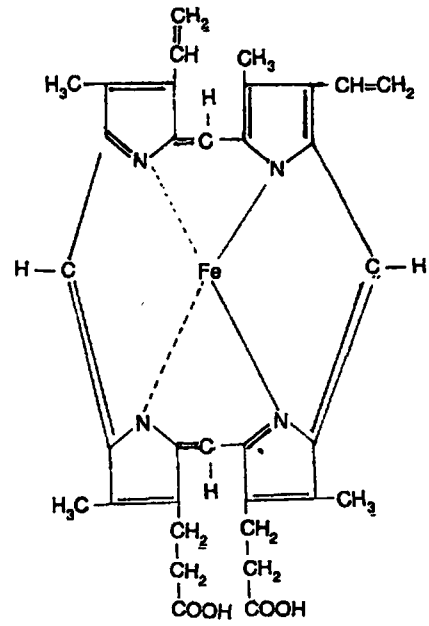


Fig. 13.10. Structure of protohaem.

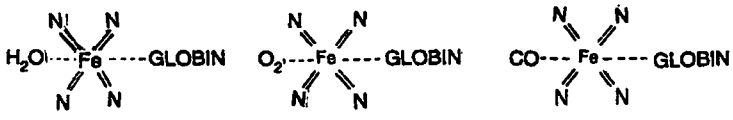
Globin has four polypeptide chains and in humans the chains are mainly of two types.

1. α-chains
2. β-chains

α-Chains have valyl-leucyl as end group. In a normal adult, haemoglobin has two α-chains; each α-chain is having 141 amino acids.

β-Chains have valyl-histidyl-leucyl as end group. In a normal adult, haemoglobin has two β-chains; each β-chain is having 146 amino acids.

The structure of haem is similar to chlorophyll. It is made up of four pyrrole groups. In this case an iron atom instead of magnesium is present in the centre of the molecule (Fig. 13.10). The iron atom is in conjugation with the protein part of the molecule.



Haemoglobin (reduced form) Oxyhaemoglobin CO-haemoglobin

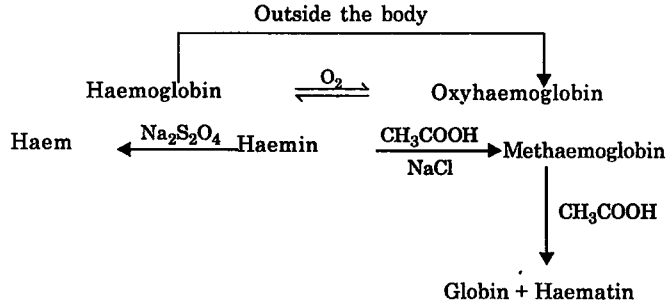
In the animal body, haemoglobin readily undergoes combination with oxygen forming unstable oxyhaemoglobin. This unstable compound loses oxygen when it reaches the various parts of the organism. In this way haemoglobin acts as a carrier of oxygen from the organs of respiration to the tissues where it takes part in various metabolic reactions.

Carbon monoxide acts as a poisonous substance because it forms a compound with haemoglobin. This compound is more stable than oxyhaemoglobin. Thus, carbon monoxide is able to displace oxygen in the blood, thus preventing the tissues to receive oxygen for carrying out their various metabolic reactions. It may lead to the death of the organism.

$$\text{More stable compound than oxyhaemoglobin} \xleftarrow{CO} \text{Haemoglobin} \underset{}{\overset{O_2}{\rightleftharpoons}} \text{Oxyhaemoglobin}$$

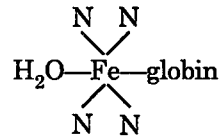
When oxyhaemoglobin is present outside the organism, it is changed into a stable compound called methaemoglobin which on treatment with acetic acid yields globin and a brownish-red

pigment haematin $[C_{34}H_{32}N_4O_4Fe(III)]^+$. OH^-. However, if methaemoglobin is treated with acetic acid having sodium chloride, it gets hydrolysed, forming haemin and globin.



It is to be remembered that iron free and iron containing compounds are called porphyrins and haems respectively.

The oxygen clarrying properties of haemoglobin has been inhibited by certain chemicals like nitrophenols, chlorates, aniline etc., which do not bind with the iron atom but they change it from ferrous state to ferric state. The compound thus formed has brown colour and has been termed as *methaemoglobin*. Methaemoglobin cannot be oxygenated and the oxygen carrying properties of the haemoglobin will therefore be lost.



Methaemoglobin

14 Chemistry of Lipids

14.1 Introduction

Lipids comprise a heterogeneous group of compounds which are sparingly soluble in water but show considerable solubility in organic solvents such as ether, chloroform, benzene, hot alcohol and petroleum ether, and on hydrolysis yield fatty acids which are utilised by the living organisms. However, the term 'lipid' includes any biological compound that is soluble in a 'lipid' (*i.e.*, non-polar organic) solvent. A widely-used 'universal' lipid solvent is chloroform + methanol (2 : 1, v/v).

As lipids are soluble in organic solvents, this shows the hydrophobic nature of the hydrocarbon structure although lipids may also contain some hydrophilic groups.

The lack of solubility of lipids in water is an important property because our body chemistry is firmly based on water. Most body constituents, including carbohydrates, are soluble in water. But the body also needs compounds that are insoluble, for many purposes, including the separation of compartments containing aqueous solutions from each other, and that is where lipids come in.

The water-insolubility of lipids is due to the fact that the polar groups they contain are much smaller than their alkane-like (nonpolar) portions. These non-polar portions provide the water-repellent, or hydrophobic, property.

14.2 Occurrence of Lipids

Lipids are widely distributed throughout the plant and animal kingdom. In plants, they occur in the seeds, nuts and fruits. In animals, they are stored in adipose tissues, bone marrows and nervous tissues.

14.3 Biological Functions of Lipids

The various biological functions of lipids are as follows :

1. The most important role of lipids is as a fuel. Much of the carbohydrates of the diet is converted to fat which is stored in various tissues and utilised at the time of requirement. Thus fat may be the major source of energy for many tissues. Actually, in some respects lipids (fats) are even superior to carbohydrates as source of energy.

(*i*) Fat yields more heat per gm when burnt. Although our bodies do store some carbohydrates in the form of glycogen for quick energy when we need it, energy stored in the form of fats is much more important. The reason is simply that the burning of fats produces more than twice as much energy (about 9 kcal/g) as the burning of an equal weight of carbohydrates (about 4 kcal/g).

(*ii*) Fat can be stored in practically anhydrous condition and in almost unlimited quantities. Thus fat is the most concentrated form in which potential energy can be stored.

(*iii*) Fatty acids with their flexible backbones can be stored in a much more compact form than the highly spatially oriented and rigid glycogen structure. Thus fat storage provides economy in both weight and space.

(*iv*) As fat is insoluble in water, once it has been carried to the fat depots by the specialised transport proteins in the plasma it is unlikely to break loose and go in the watery body fluids which bath the adipose tissue.

(*v*) Fat remains as a stable and fixed reserve of energy until mobilized by enzymes which hydrolyse it to glycerol and fatty acids. The enymes are under the control of various hormones and are activated under conditions where the body is involved in increased energy expenditure.

In spite of the above advantages, carbohydrate, and not fat, is the preferred fuel of the body, and any attempt to oxidize appreciable quantities of fat without concomitant degradation of adequate amount of carbohydrate can lead to serious consequences.

2. Since fat is a bad conductor of heat, it provides excellent insulation. Thus in cold conditions, in which heat is lost to the environment, it provides both an insulating blanket and an extra energy source.

3. Fat may also provide padding to protect the internal organs. Brain and nervous tissue are rich in certain lipids, a fact which indicates the importance of these compounds to life.

4. Some compounds derived from lipids are important building blocks of biologically active materials, *e.g.*, acetic acid can be used by the body to synthesize cholesterol and related compounds (hormones).

5. Lipoproteins are constituents of cell walls. The lipids present in lipoproteins constituting the cell wall are of the types of phospholipids. Since lipids are water insoluble they act as ideal barrier for preventing water soluble materials from passing freely between the intra and extra-cellular fluids.

6. One more important function of dietary lipids is that of supplying the so called essential fatty-acids. Although there are several functions of essential fatty acids (EFA), none of them is well defined.

(*i*) EFA are found to be the constituents of structural lipids of the cell and mitochondrial membrane.

(*ii*) These are also found in the reproductive organs in high concentration.

(*iii*) EFA are also present in phospholipids in the 2-position.

(*iv*) Arachidonic acid and related C_2 fatty acids give rise to a group of pharmacologically active compounds.

(*v*) EFA are also involved in the genesis of fatty livers and in the metabolism of cholesterol.

(*vi*) In infants certain types of eczema have been cured by feeding fats containing the essential fatty acids.

7. Dietary fat is also found to be necessary for the sufficient absorption of the essential fatty acid and fat-soluble vitamins from the gastro intestinal tract.

However, it is important to note that deficiency of an essential fatty acid has not been observed in man, because the amounts required are very small and absolutely fat free diet is practically unknown.

14.4 Classification of Lipids

Lipids have been generally classified into simple lipids, compound (*conjugated*) lipids and steroids. Let us describe these one by one.

14.5 Simple Lipids

Simple lipids are esters of fatty acids with alcohols. Examples of simple lipids are neutral fats and waxes.

I. Neutral fats : They are the esters of fatty acids with glycerol. They are also called triglycerides because three molecules of fatty acids condense with one mole of glycerol to form fat. For example, three molecules of butyric acid on condensation with one molecule of glycerol yield tributyrin, a fat. Similarly, palmitic acid condenses with glycerol to form tripalmitin, a fat.

$$\begin{array}{l} CH_2OH \\ CHOH \\ CH_2OH \end{array} + \begin{array}{l} HOOCCH_2CH_2CH_3 \\ HOOCCH_2.CH_2CH_3 \\ HOOCCH_2.CH_2CH_3 \end{array} \longrightarrow \begin{array}{l} CH_2O.CO.CH_2CH_2CH_3 \\ CHO.CO.CH_2CH_2CH_3 \\ CH_2O.CO.CH_2CH_2CH_3 \end{array}$$

Tributyrin

The neutral fats on hydrolysis yield fatty acids and glycerol. This hydrolysis is carried out by lipases.

$$\begin{array}{l} CH_2—O—CO—R^1 \\ CH_2—O—CO—R^2 + 3H_2O \\ CH_2—O—CO—R^3 \end{array} \overset{H^+}{\rightleftharpoons} \begin{array}{l} CH_2OH \quad HOOC—R^1 \\ CHOH + HOOC—R^2 \\ CH_2OH \quad HOOC—R^3 \end{array}$$

Triglyceride — Glycerol — 3 Fatty acids

When the three fatty acids of a triglyceride are identical the triglyceride is said to be *simple*. When the fatty acids are not identical the triglyceride is said to be a *mixed* triglyceride.

Natural fats are mainly composed of mixed glycerides. As these glycerides have no free acid or basic groups, they are often termed as neutral fats.

At room temperatures, fats are solids or liquids (oils). Fats are lighter than water, sp. gr. 0.86. The melting point of the fat has been found to depend upon chain length and degree of saturation of fatty acids. The melting points of fats have been found to be always higher than their solidification points. For example, tristearin melts at 72°C but solidifies on cooling at 52°C.

Fatty acids : Naturally-occurring fatty acids mostly have even number of carbon atoms,

and except for those of bacterial origin, they might be highly unsaturated.

The reason that only even-numbered acids are found in fats is that the body builds these acids entirely from acetic acid units and therefore put the carbons in two at a time.

As all the fats contain glycerol, their properties have been found to differ according to the nature of fatty acids present in them. Fatty acids occurring in higher animals tend to have chain lengths of 16,18 or 20 carbon atoms except in milk fat, where the saturated fatty acids C_{10}—C_{14} make up 10% of the molar total. Oleic acid is the most abundant fatty acid in humans, both in depot fat and in milk. Butyric acid with four carbon atoms is normally the shortest fatty acid found in human fat.

The general formula of fatty acids is $CH_3\ (CH_3)_n\ COOH$ or RCOOH, where *R* is a hydrocarbon chain. The $CH_3\ (CH_2)_n$ CO-chain is termed as an acyl radical. The value of *n varies* from zero in acetic acid to 86 in mycolic acid.

The carboxylic acid (—COOH) group of a fatty acid is strongly polar. It ionizes in water at intracellular pH by the loss of a proton.

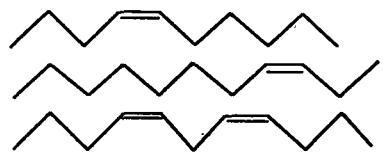
$$RCOOH \rightleftharpoons RCOO^- + H^+$$

The hydrocarbon chain is insoluble in water and is *non-polar*. Fatty acids therefore possess *hydrophilic* as well as *hydrophobic* character. At physiological pH fatty acids exist in solution as the ionized alkyl carboxylates, R—COO^-, *e.g.*, *stearate* : $CH_3(CH_2)_{18}$—COO^-.

The fatty acids can be divided into two groups : *saturated* and *unsaturated*. Saturated fatty acids have only single bonds in the hydrocarbon chain. Unsaturated fatty acids have at least one C = C double bond in the chains. All the unsaturated fatty acids listed in Table 14.1 are the cis isomers. This explains their physical properties, reflected in their melting points. Saturated fatty acids are solids at room temperature because the regular nature of their aliphatic chains allows the molecules to be packed in a close, parallel alignment.

The interactions (van der Waals forces) between neighbouring chains are weak, but the regular packing allows these forces to operate over a large portion of the chain so that a considerable amount of energy is needed in order to melt them.

In contrast, unsaturated fatty acids are all liquids at room temperature because the cis double bond interrupts the regular packing of the chains.



Thus much less energy is required to melt them. The greater the degree of unsaturation, the lower the melting point because each double bond introduces more disorder into the packing of the molecules.

A saturated fatty acid is having maximum possible number of attached hydrogens. Each carbon atom has two hydrogen atoms attached to it. One of the terminal carbon atom is having three hydrogen atoms while the other is having a carboxyl (—COOH) group. The hydrocarbon

portions of saturated fatty acids have only single bonds. Examples of saturated fatty acids are listed in Table 14.2.

Table 14.1
MAJOR SATURATED FATTY ACID FOUND IN PLANTS AND ANIMALS

Name	*Formula*	*Common source*
Butyric acid	$CH_3(CH_2)_2COOH$	butter
Caproic acid	$CH_3(CH_2)_4COOH$	butter
Caprylic acid	$CH_3(CH_2)_6COOH$	coconut oil, palm oil
Lauric acid	$CH_3(CH_2)_{10}COOH$	laurel kernel oil
Myristic acid	$CH_3(CH_2)_{12}COOH$	nutmeg oil
Palmitic acid	$CH_3(CH_2)_{14}COOH$	palm oil, animal fat
Stearic acid	$Ch_3(CH_2)_{16}COOH$	cocoa butter
Arachidic acid	$CH_3(CH_2)_{18}COOH$	peanut oil

Unsaturated fatty acids have one or more double bonds. The general formula for these fatty acids is R—CH = CH $(CH_2)_n$COOH. Generally the double bonds range between 1 to 6 and occur after 9,12,15,18, etc. carbon atoms. Some common unsaturated fatty acids are listed in Table 14.2.

Table 14.2
MAJOR UNSATURATED FATTY ACIDS IN PLANTS AND ANIMALS

Name	*Formula*	*Source*
Palmitoleic acid	$CH_3(CH_2)_5CH = CH(CH_2)_7COOH$	milk fat, sardine oil
Oleic acid	$CH_3(CH_2)_7CH = CH\ (CH_2)_7COOH$	olive oil, pork fat
Linoleic acid	$CH_3(CH_2)_4CH = CHCH_2CH = CH(CH_2)_7COOH$	linseed oil, soyabean oil
Linolenic acid	$CH_3CH_2CH = CHCH_2CH = CHCH_2CH = CH(CH_2)_7COOH$	lineseed and hempseed oil
Erucic acid	$CH_3(CH_2)_7CH = CH(CH_2)_{11}COOH$	mustard oil

Fatty acids having more than one double bond are termed as *poly-unsaturated* fatty acids. According to the number of double bonds, fatty acids are termed as *monoenoic* (1 double bond), *dienoic* (2 double bonds), *trienoic* (3 double bonds), *tetraenoic* (4 double bonds) etc.

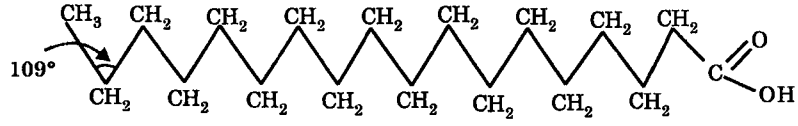
Bacteria do not have polyunsaturated fatty acids. Their principal fatty acid (*vaccenic acid*) is monoenoic.

Unsaturated fats have been found to be more common in living organisms than saturated fats. Unsaturated fats may have an *odd* or an *even* number of carbon atoms. *Odd-numbered* fatty acids occur in all cells as minor components of total fatty acids. They are more common in plants, but are also found in animals. Most of the fatty acids in animals are *even-numbered.* This is to be expected because animal cells synthesize fatty acids by polymerization of 2-C units.

Increase in the number of double bond progressively decreases the melting point. In general, the melting points of unsaturated fatty acids are lower than saturated fatty acids and are oils rather than solids at room temperature. Plant triglycerides have a large proportion of *unsaturated*

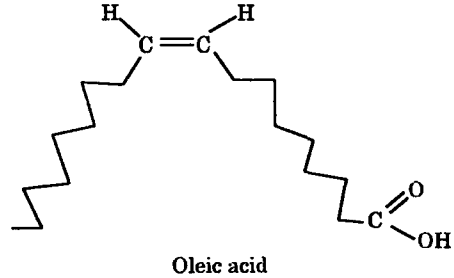
fatty acids such as *oleic, linoleic* and *linolenic acids.* They therefore have low melting points and are liquids at room temperature. Animal triglycerides on the other hand have a higher proportion of saturated fatty acids such as *palmitic and stearic acids.* They have higher melting points and are solids or semisolids at room temperature.

The hydrocarbon chain of a saturated fatty acid possesses zigzag configuration with the bond angle between carbon-carbon being 109°. Hence *stearic acid* (18C) can be depicted as follows :



Stearic acid

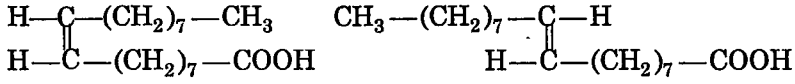
Introduction of a double bonds, *e.g.*, that between carbon 9 and carbon 10 in the *oleic acid* molecule, causes a bend in the molecule.



Oleic acid

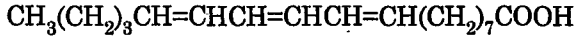
Introduction of two double bond (*e.g., linoleic acid*) causes further bending of the hydrocarbon chain.

Geometric isomerism occurs in fatty acids whose hydrocarbon chains have double bonds. Most unsaturated fatty acids occur in the relatively less stable *cis* isomeric form rather than more stable *trans* form.



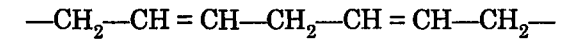
Oleic acid (*cis*) Elaidic acid (*trans*)

Polyunsaturated fatty acids might possess a *conjugated* or a *non-conjugated* double bond system. α-*Elaeostearic acids* has a *conjugated* triene double bond system.



The conjugated double bond system has been found to be more reactive leading to polymerization of fatty acids.

Linoleic acid possesses a *nonconjugated* double bond system



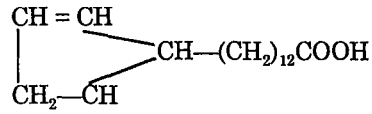
Linoleic acid with nonconjugated double bond system.

In the non-conjugated system the methylene group is having double bonds on both sides.

In some animal fats and in bacteria, branched chain fatty acids also occur. They generally have odd number of carbon atoms (C_{13} to C_{17}). Tuberculostearic acid is an example, which occurs in *Bacillus tuberculosis.*

$$CH_3—(CH_2)_6—\underset{\displaystyle CH_3}{\overset{\displaystyle |}{CH}}—(CH_2)_8COOH$$

Fatty acids having cyclic systems also occur in nature. An example is chaulmoogric acid which is obtained from chaulmoogra (*Hydnocarpus wightiana*) oil.



Chaulmoogric acid

Linoleic, linolenic and arachidonic acids, which are unsaturated acids, also occur in small amounts in animal fats. These three are sometimes termed as essential fatty acids because they cannot be synthesized in the animal body and must be supplemented with the diet.

Physical state : With some exceptions, fats that come from animals are generally solids at room temperature, and those from plants or fish are usually liquids. Liquid fats are often called oils, though they are esters of glycerol just like solid fats and should not be confused with petroleum, which is mostly alkanes.

What is the structural difference between solid fats and liquid oils? In most cases it is the degree of unsaturation. The physical properties of the fatty acids are carried over to the physical properties of the triglycerides. Solid animal fats contain mainly saturated fatty acids, and vegetable oils contain high amounts of unsaturated fatty acids. Table 14.3 shows the average fatty acid contents of some common fats and oils. Note that even solid fats contain some unsaturated acids and that liquid fats contain some saturated acids. Some unsaturated fatty acids (linolenic and linolenic acids) are called essential fatty acids since the body cannot synthesize them from precursors and they must therefore be included in the diet.

Though most vegetable oils have high amounts of unsaturated fatty acids, there are exceptions. Note that coconut oil has only a small amount of unsaturated acids. This oil is a liquid not because it contains many double bonds but because it is rich in low-molecular-weight fatty acids (chiefly lauric).

Table 14.3
AVERAGE PERCENTAGE OF FATTY ACIDS AND IODINE NUMBER OF SOME COMMON FATS AND OILS

	Lauric	*Myristic*	*Palmitic*	*Stearic*	*Oleic*	*Linoleic*	*Linolenic*	*Other*	*Iodine Number*
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Animal Fats									
Beef tallow	—	6.3	27.4	14.1	49.6	2.5	—	0.1	50
Butter	2.5	11.1	29.0	9.2	26.7	3.6	—	17.9	36
Human	—	2.7	24.0	8.4	46.9	10.2	—	7.8	68
Lard	—	1.3	28.3	11.9	47.5	6.0	—	5.0	59
Vegetable Oils									
Coconut	45.4	18.0	10.5	2.3	7.5	—	—	16.3	10
Corn	—	1.4	10.2	3.0	49.6	34.3	—	1.5	123
Cottonseed	—	1.4	23.4	1.1	22.9	47.8	—	3.4	106

(Contd.)

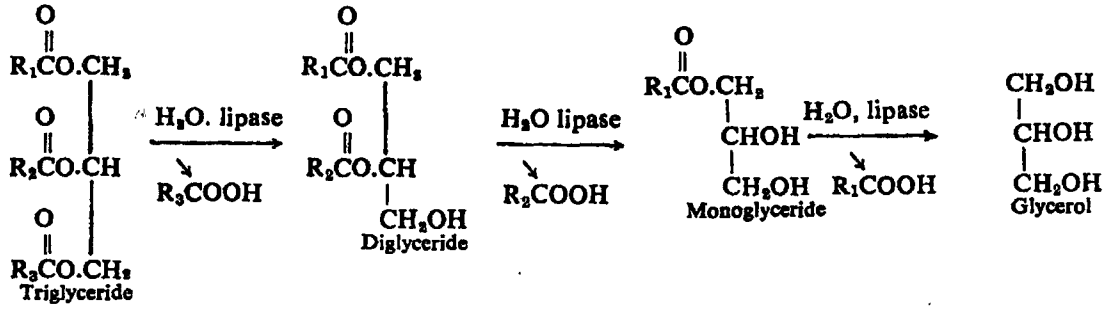
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Linseed	—	—	6.3	2.5	19.0	24.1	47.4	0.7	179
Olive	—	—	6.9	2.3	84.4	4.6	—	1.8	81
Palm	—	1.4	40.1	5.5	42.7	10.3	—	—	54
Peanut	—	—	8.3	3.1	56.0	26.0	—	6.6	93
Safflower	←	6.8	→		18.6	70.1	3.4	1.1	145
Soyabean	0.2	0.1	9.8	2.4	28.9	52.3	3.6	2.7	130

Oils with an average of more than one double bond per fatty acid chain are called polyunsaturated. For some years there has been a controversy about whether a diet rich in unsaturated and polyunsaturated fats helps to prevent heart attacks.

Pure fats and oils are colourless, odourless, and tasteless. This statement may seem surprising since we all know the tastes and colours of such fats and oils as butter and olive oil. The tastes, odours, and colours are caused by substances dissolved in the fat or oil.

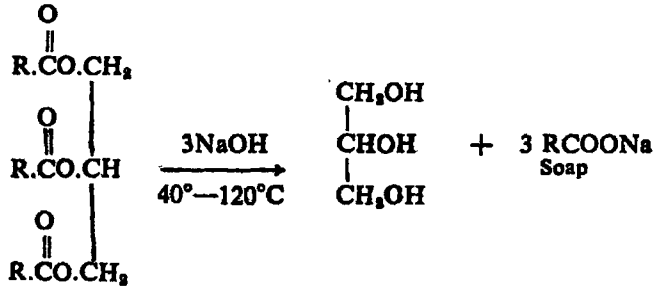
Chemical Properties of Fats

(a) Hydrolysis : When fats are hydrolysed with alkali or enzyme lipase, they yield fatty acids and glycerol. Lipases bring about splitting of fats in steps, from triglyceride to diglyceride to mono-glyceride and finally to glycerol and fatty acids. Lipases may work in the temperature range of 0° to 40°C.



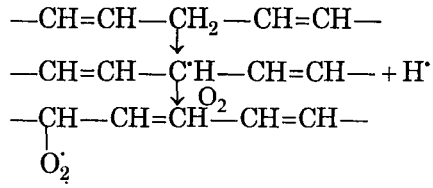
The fats are hydrolysed with alkali yielding the free fatty acids which react with alkali to form salts. These salts are soaps and this process is termed as saponification.

(b) Oxidation : Oxidation of fats in the air takes place along with hydrolysis. Oxidation takes place at double bonds of the fatty acid, yielding short chain acids, aldehydes etc. This along with the fatty acids formed during hydrolysis imparts a less palatable taste and odour to the fat. It is



termed as *rancidity*. Oxidation can be prevented by using small amount of certain organic substances called antioxidants. Commercially antioxidants used are nordihydroguiretic acid and tertiary butyl *para*-cresol. Tocopherol is a natural antioxidant which occurs in vegetable oil.

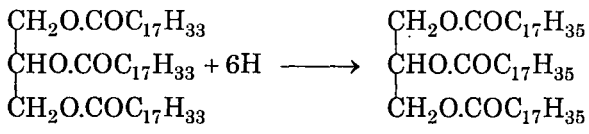
In biochemical terms, oxidation means direct attack by O_2. The initial product is generally a peroxide.



The initial radical can then activate another acyl chain and so propagate the reaction (Note the shift of the double bond).

(c) Hydrogenation : By calaytic hydrogenation, unsaturated plant fats get converted into more saturated and solid fats. It is generally done over finely divided nickel. In the production of margarine and vegetable shortening, this property has been exploited commercially.

$$\text{Vegetable oil} \xrightarrow[\text{Ni powder pressure}]{H_2 \text{ gas}} \text{Solid fat}$$



Triolein (oil) Tristearin (fat)

This hydrogenation is carried out on a large-scale to produce the solid shortening sold in stores under various brand names. In making such products, manufacturers must be careful not to hydrogenate all the double bonds because a fat with no double bonds at all would be too brittle. Partial, but not complete, hydrogenation results in a product with the right consistency for cooking.

Rancidity : The double bonds in fats and oils are subject to oxidation by the air. When a fat or oil is allowed to stand out in the open, this reaction slowly turns some of the molecules into aldehydes and other compounds with bad tastes and odours. We say that the fat or oil has become rancid and is no longer edible. Vegetable oils, which generally contain more double bonds, are more susceptible to this than solid fats, but even fats contain some double bonds, so rancidity can be a problem here, too.

Another cause of unpleasant taste is hydrolysis which is brought about by certain enzymes like lipase which is mainly present in naturally occurring fats, especially animal fats. The hydrolysis of triglycerides may produce short-chain fatty acids, which have bad odours. In order to prevent rancidity, fats and oils should be kept refrigerated (these reactions are slower at low temperatures) and in dark bottles (the oxidation is catalyzed by ultraviolet light). In addition to this, antioxidants are often added to fats and oils to prevent rancidity.

It is to be noted that the vegetable fats contain certain substances like vitamin E, vitamin C, phenols, hydroquinone, tannins, etc. which are antioxidants and therefore prevent development of rancidity. Hence vegetable fats can be preserved for a longer period than animal fats.

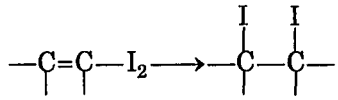
Characterization of fats : The composition, quality and purity of a given oil or fat is determined by means of a number of physical and chemical tests. The usual physical tests include

determination of m.p., specific gravity, viscosity, etc. while the chemical tests include determination of certain values discussed below.

1. Acid number : It is the number of milligrams of potassium hydroxide required to neutralise the free fatty acids in 1 g. of the oil or fat. Thus it indicates the amount of free fatty acids present in an oil or fat. A high acid value indicates a stable oil or fat stored under improper conditions.

2. Saponification number : It is the number of milligrams of potassium hydroxide required to completely saponify 1 g of the oil or fat. Thus it is a measure of fatty acids present as esters in a given oil or fat. The saponification value gives an idea about the molecular weight of fat or oil. The saponification number and molecular weight of an oil are inversely proportional to each other; thus high saponification number indicates that the fat is made up of low molecular weight fatty acids and *vice versa*. It is also helpful in detecting adulteration of a given fat by one of the lower or higher saponification value.

3. Iodine number : It is the number of grams of iodine that combine with 100 g of oil or fat. It is a measure of the degree of unsaturation of a fat or oil; a high iodine number indicates a high degree of unsaturation of the fatty acids of the fat. It is useful to have a test for degree of unsaturation in fats and oils. One such test which has been used for many years is the iodine number test. Recall that halogens add on to the carbons of a double bond.



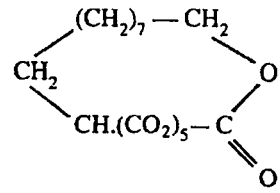
The test consists of adding a halogen to a sample of fat or oil. Iodine numbers for some common fats and oils are given in Table 14.4. As the table shows, liquid oils generally have higher iodine numbers that solid fats.

4. Reichrt-Meissl number : (R.M. number) : It is the number of millilitres of N/10 potassium hydroxide required to neutralise the distillate (obtained by saponification, acidification and steam distillation of the fat) of 5g of the fat. It is a measure of steam volatile fatty acids present as esters in oil or fat. It is used for determining the purity of butter or ghee (R.M. number 20-30).

Table 14.4
VALUE OF CONSTANTS FOR SOME OF THE COMMON FATS

Fat	*Acid No.*	*Saponi No.*	*Iodine No.*	*R.M. No.*
Human fat	—	196	65	0.40
Beef fat	0.25	198	40	—
Butter fat	0.40	220	27	26.0
Linseed oil	2.2	192	190	1.0
Coconut oil	1.5	258	8	7.0

II. Waxes : They are esters of high molecular weight fatty acids with alcohols other than glycerol (true waxes) or with sterols such as cholesterol (steryl esters). The constituent fatty acids and alcohols have usually 24–36 carbon atoms. Carnauba wax is composed of myricyl alcohol (30C) and cerotate (26C). In bees wax, the fatty acid constituent is a smaller chain acid, palmitic acid (16C), and alcohol is myricyl. Ambretolide which occurs in the seeds of *Abelmoschus esculentus* is a hydroxy acids. This acid is responsible for the characteristic smell of the seed.



Ambretolide

Some of the important alcohols and acids found in waxes are tabulated in Table 14.5.

Table 14.5

Alcohols		*Acids*	
Lauryl	$C_{12}H_{25}OH$	Myristic	$C_{13}H_{27}COOH$
Cetyl	$C_{16}H_{33}OH$	Palmitic	$C_{15}H_{31}COOH$
Octodecyl	$C_{18}H_{37}OH$	Cerotic	$C_{25}H_{51}COOH$
Caranaubyl	$C_{24}H_{49}OH$	Melissic	$C_{29}H_{59}COOH$
Ceryl	$C_{26}H_{53}OH$		
Myricyl	$C_{30}H_{61}OH$		
Cocceryl	$C_{30}H_{60}(OH)_2$		

In some waxes, the alcohol component may be cholesterol. Such waxes are found in blood.

As waxes have fully reduced hydrocarbon chains, they are insoluble in water and very resistant to atmospheric oxidation. Their melting points are very high. Because of these properties they find uses in furniture polishing. They are chemically inert and not digested by the fat splitting enzymes. They are slowly splitted with hot alcoholic KOH.

Waxes generally have higher melting points than fats (60° to 100°C) and are harder. Animals and plants use them for protective coatings. The leaves of most plants are coated with wax, which helps to prevent microorganisms from attacking them and also allows them to conserve water. The feathers of birds and the fur of animals are also coated with wax. This is what allows ducks to swim. The ears of humans are protected by ear wax.

Waxes are saponified with great difficulty than fats and are not attacked by lipase. Although waxes may be saponified by prolonged boiling with alcoholic KOH, they are more easily saponified by treating a solution of the wax in petroleum ether with absolute alcohol and metallic sodium, *i.e.*, with sodium ethoxide. The saponification products of waxes are water-soluble soaps (sodium salts of higher fatty acids); while the water insoluble long-chain alcohols appear in the "unsaponifiable matter" fraction. Waxes contain about 31-55% of the unsaponifiable matter, while fats and oils contain only 1-2% unsaponifiable matter.

The waxes serve as water barriers in insects, birds and furred animals. Waxes also serve as protective coating on fruits and leaves. Lanonin is secreted in the skin of most fur-bearing animals.

Some important waxes are carnauba wax (from a Brazilian palm tree), lanolin (from lamb's wool) beeswax, and spermaceti (from whales). These are used to make cosmetics, polishes, candles, and ointments. Paraffin waxes are not esters. They are mixtures of high-molecular weight alkanes.

Common waxes

Beeswax : It contains esters derived from alcohols having 24-30 carbon atoms, *viz.*, palmitate of myricyl alcohol ($C_{30}H_{61}OH$) and *n*-hexacosanol ($C_{26}H_{53}OH$).

$CH_3(CH_2)_{14}.COOC_{30}H_{61}$
Myricyl palmitate

$CH_3.(CH_2)_{14}COOC_{26}H_{53}$
n-Hexacosanyl palmitate

Spermaceti : It is obtained from the head of the sperm whale. It is rich in ester of cetyl alcohol ($C_{16}H_{38}OH$) and palmitic acid.

$CH_3.(CH_2)_{14}.COOC_{16}H_{33}$
Cetyl palmitate

Spermaceti is used in making candles.

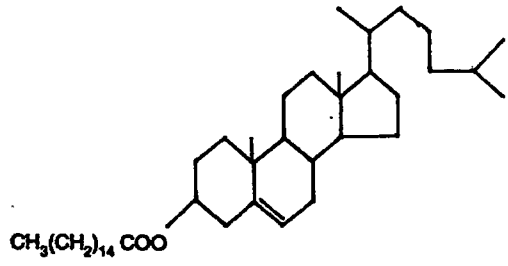
Sperm oil : It is a liquid wax and occurs with spermaceti in the sperm whale. It is a valuable lubricant used for delicate instruments, such as watches. It does not become gummy, as many oils do.

Carnauba wax : It is found in the leaves of the carnauba palm of Brazil. It is used as an ingredient in the manufacture of various wax polishes because waxes are very inert chemically, they make an excellent protective coating.

Lanolin or wool wax : It is obtained from wool and is used in making ointments and salves. It readily forms an emulsion with water, and for this reason makes it possible for drugs which are soluble in water to be incorporated into salves. Chinese wax is the secretion of an insect.

Some of the waxes found in skin are esters of hydroxylated fatty acids and open chain alcohols.

A wax found in blood plasma is found to have cholesteryl plamitate, *i.e.*, ester of cholesterol and palmitic acid.



Cholesteryl palmitate

Physiological Importance of Waxes

(*i*) The most important physiological function of waxes is as a protective agent on the surface of animals and plants.

(*ii*) Waxes are found on the surface of feathers and hair with the result they remain soft and pliable.

(*iii*) Waxes prevent aquatic animals from becoming wet. On coming out of water a duck will shake herself once and becomes apparently dry.

(*iv*) Waxy coating on the surface of plants protects them from excessive loss of moisture. Hence desert plants like palm and cactus can live for long periods without rain.

(*v*) Waxy coating protects the plant from becoming infected with fungi and bacteria which cause disease.

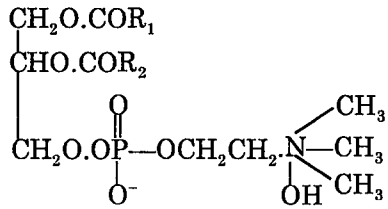
(*vi*) Waxy coating on several fruits like apples and citrus fruits prevent them from dying out and thus these fruits can be stored for long periods of time. The waxy covering also protects such fruits from organisms which cause rot.

14.6 Compound Lipids

The lipids having some additional groups or elements besides fatty acids and alcohol are called compound lipids. The additional group may have phosphorus, nitrogen, sulphur, or it may be a protein. The various types of compound lipids are as follows :

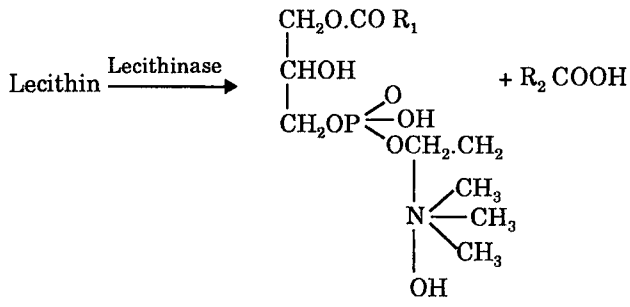
(a) ***Phospholipids :*** These are the lipids having glycerol, phosphoric acid and fatty acids. These may be regarded as fats in which one of the fatty acids has been replaced by a phosphoric acid, Phospholipids are of the following types :

(i) Lecithins (Phosphatidyl cholines) : Lecithins occur widely in nature. When hydrolysed, they yield glycerol, fatty acids, phosphoric acid and choline. They are also called phosphatidyl cholines. The fatty acids commonly occurring in lecithin are palmitic, stearic, oleic, linolenic and arachidonic acids.



Lecithin

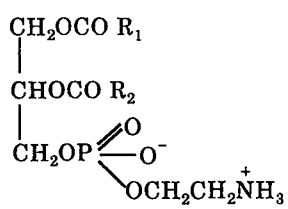
Lecithins are yellowish grey solids. They are soluble in ether and alcohol but insoluble in acetone. When exposed to air, they rapidly darken in colour and absorb water, forming dark greasy mass. Lecithins are broken down by the enzyme lecithinase to lysolecithin.



Lysolecithin

The enzyme lecithinase occurs in venoms of bee and cobra. When it is injected into the blood, lysolecithins bring about rapid haemolysis of the red blood cells.

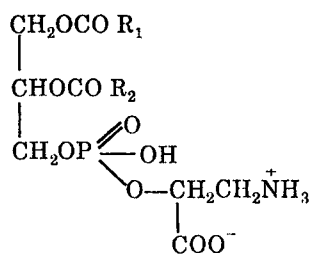
Lecithin is an important constituent of lipoproteins, particularly chylomicrons. Egg yolk is a rich source of lecithin, which also has an important role in fat metabolism in the liver. At physiological pH the positive and negative groups of lecithin are equally ionized and there is no net charge. Therefore, it is a *neutral* phospholipid. The lecithins in human RBC membranes are reported to exist in more than 20 forms.



Cephalin (phosphatidyl ethanolamine)

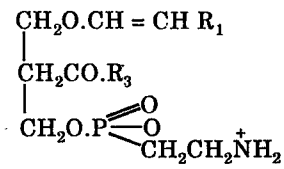
(ii) Cephalins (Phosphatidyl enthanolamines) : They occur in animal tissues in close association with lecithins. They also occur in soyabean oil. The main difference between the lecithins and cephalins is the nature of nitrogenous base. Cephalins have ethanolamine, colamine and some times serine in place of choline. The fatty acid components of cephalins are generally stearic, oleic, linoleic and arachidonic acids.

Phosphatidyl serine (cephalin) occurs in lesser amount in all issues. It is an acid phospholipid because it carries a net negative charge at physiological pH. It has serine as the polar compound.



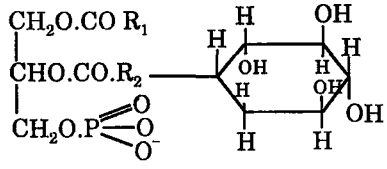
Photphatidyl serine

(*iii*) *Plasmalogens* : They occur abundant in brain and muscles. They also occur in the seeds of higher plants. In this group of phospholipids, a complex aldehyde is attached to the α-carbon atom of glycerol. Rest of the molecule has been found to be similar to lecithin or cephalin. Plasmalogens dissolve in all the lipid solvents.

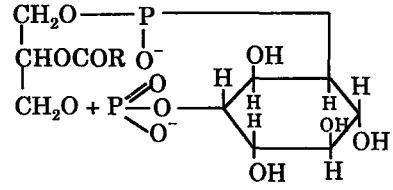


(or choline)
Plasmalogen

(*iv*) *Phosphoinostitides* : Phosphoinostides have hexahydric alcohol inositol. They can be either mono or diphosphoinositides. The name lipoinositol was also postulated for these substances.



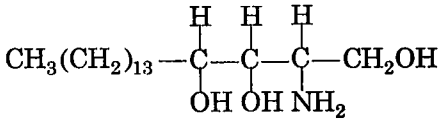
Monophosphoinositides



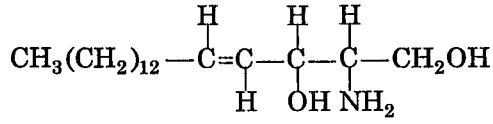
Diphosphoinositide

Monophosphoinositides are widely distributed in animals and plants but diphosphoinositides have been reported to be present in brain tissues only.

(*v*) *Phophosphingosides* : These are the lipids in which glycerol is replaced by either sphingosine or phytosphingosine. These lipids occur in nervous tissues and apparently lack in plants and microorganisms.

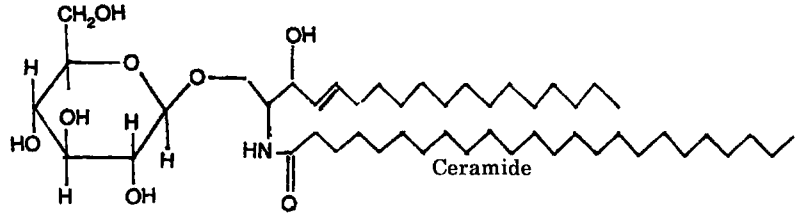


Phytosphingosine



Sphingotine

(b) Glycolipids : In cerebrosides the fatty acid of the ceramide part may contain either 18-carbon or 24-carbon chains, the latter found only in these complex lipids. A glucose or galactose carbohydrate unit forms a beta glycosidic bond with the ceramide portion of the molecule. The cerebrosides occur primarily in the brain (7 percent of the dry weight) and at nerve synapses.

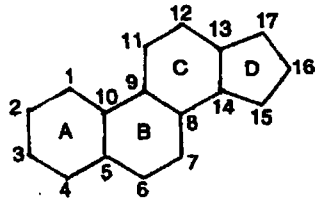


β-D-Glucose Glucocerebroside

14.7 Derived Lipids

These lipids include hydrolytic products of lipids as well as other lipid-like compounds like sterols, carotenoids, essential oils, aldehydes, ketones, alcohols, hydrocarbons etc.

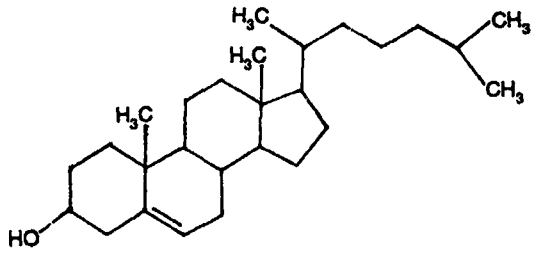
The major class of derived lipids is the steriods, which are compounds containing this ring system.



There are three cyclohexane rings (A, B and C) connected in the same way as in phenanthrene and a fused cyclopentane ring (D). Steriods are thus completely different in structure from the lipids already discussed. Note that they are not necessarily esters, though some of them are as follows.

Cholesterol

The most abundant steriod in the human body, and the most important, is cholesterol.



Cholesterol

It serves as a membrane component, mostly in the plasma membranes of red blood cells and in the myelinated nerve cells. The second important function of cholesterol is to serve as a raw material for other steriods such as the sex and adrenocorticoid hormones and bite salts.

Cholesterol exists both in the free form and esterified with fatty acids. Gallstones contain free cholesterol.

Because the correlation between high serum cholesterol and such diseases as atherosclerosis has received so much publicity, many people are afraid of cholesterol and regard it as some kind of poison. It should be apparent from this discussion that, far from being poisonous, cholesterol is necessary for human life. Without it, we would die. Fortunately, there is no chance of that, since, even if it were completely eliminated from the diet, our livers would make enough to satisfy our needs.

Cholesterol in the body is in a dynamic state. Most of the cholesterol ingested and that manufactured by the liver is used by the body to make other molecules, such as bile salts. The serum cholesterol level controls the synthesis by the liver.

At the present time, our knowledge of the role played by serum cholesterol in atherosclerosis is incomplete. The best we can say is that it probably makes good sense to reduce the amount of cholesterol and saturated fatty acids in the diet.

Steroid Hormones

Cholesterol is the starting material for the synthesis of steriod hormones. In this process, the aliphatic side chain on the D ring is shortened by the removal of a six-carbon unit, and the secondary alcohol group on C-3 is oxidized to a ketone. The resulting molecule, *progesterone,* serves as the starting compound for both the sex hormones and the adrenocorticoid hormones (Fig. 14.1).

Adrenocorticoid Hormones

The adrenocorticoid hormones are products of the adrenal glands. We divide them into two groups according to function mineralocorticoids regulate the concentrations of ions (mainly Na^+ and K^+) and glucocorticoids control carbohydrate metabolism.

Aldosterone is one of the most important mineralocorticoids. Increased secretion of aldosterone enthances the reabsorption of Na^+ and Cl^- ions in the kidney tubules and the loss of K^+. Since Na^+ concentration controls water retention in the tissues, aldosterone also controls tissue swelling.

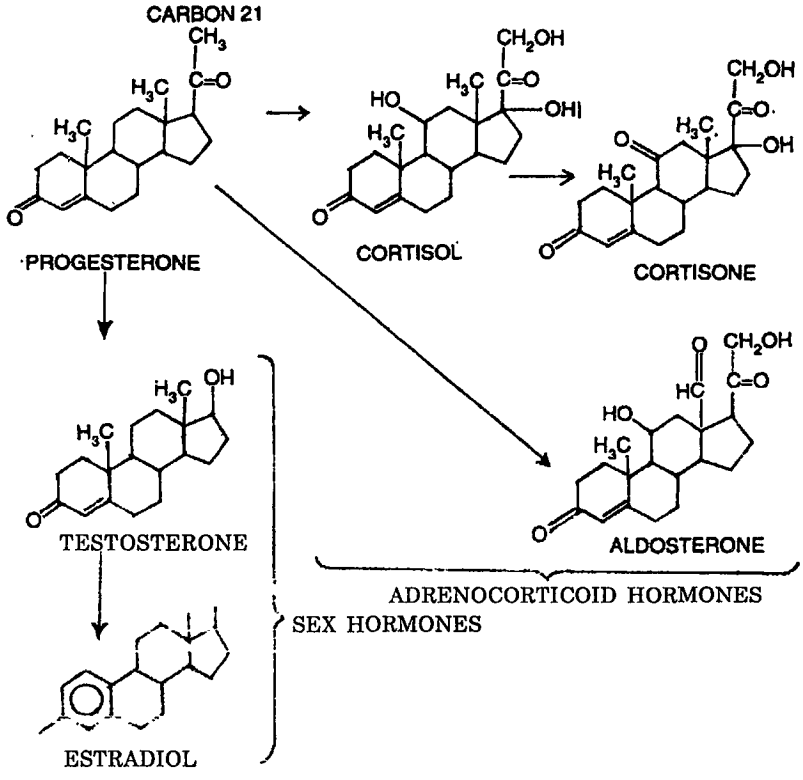


Fig. 14.1.

Cortisol is the major glucocorticoid. Its function is to increase the glucose and glycogen concentrations in the body. This is done at the expense of other nutrients. Fatty acids from fat storage cells and amino acids from body proteins are transported to the liver, which, under the influence of cortisol, manufactures glucose and glycogen from these sources.

Cortisol and its ketone derivative, *cortisone,* have remarkable antiinflammatory effects. These or similar synthetic derivatives, such as prednisolone, are used to treat inflammatory diseases of many organs, rheumatoid arthritis, and bronchial asthma.

Sex Hormones

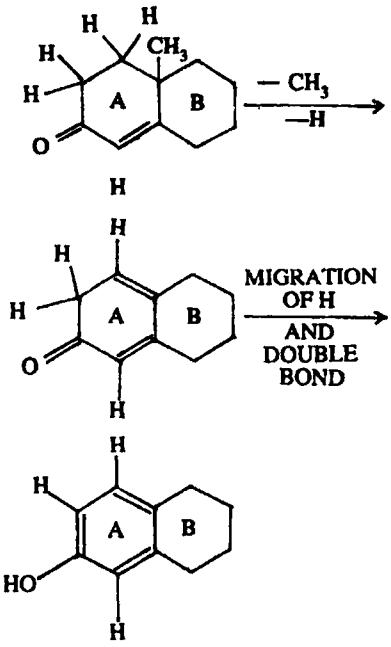
The most important male sex hormone is *testosterone* (Fig. 14.1). This hormone, which promotes the normal growth of the male genital organs, is synthesized in the testes from cholesterol. During puberty, increased testosterone production leads to such secondary male sexual characteristics as deep voice and facial and body hair.

Female sex hormones, the most important of which is *estradiol* (Fig. 14.1), are synthesized from the corresponding male hormone (testosterone) by aromatization of the A ring. Estradiol, together with its precursor progesterone, regulates the cyclic changes occurring in the uterus and ovaries known as the menstrual cycle. As the cycle begins, the level of estradiol in the body rises, and this causes the lining of the uterus to thicken. Then another hormone, called luteinizing hormone, triggers ovulation. If the ovum is fertilized, increased progesterone levels will inhibit any further ovulation. Both estradiol and progesterone then promote further preparation of the uterine lining to receive the fertilized ovum. If no fertilization takes place, progesterone production stops altogether, and estradiol production decreases. This decreases the thickening of the uterine lining, and it is then sloughed off with accompanying bleeding. This is menstruation.

Estradiol and progesterone also regulate secondary female sex characteristics, such as the growth of breasts.

Testosterone and estradiol are not exclusive to either males of females. A small amount of estradiol production occurs in males, and a small amount of testosterone production is normal in females. Only when the proportion of these two hormones (hormonal balance) is upset one can observe symptoms of questionable sexual identity.

The conversion of the male hormone to the female hormone results from the loss of a $—CH_3$ and a —H :

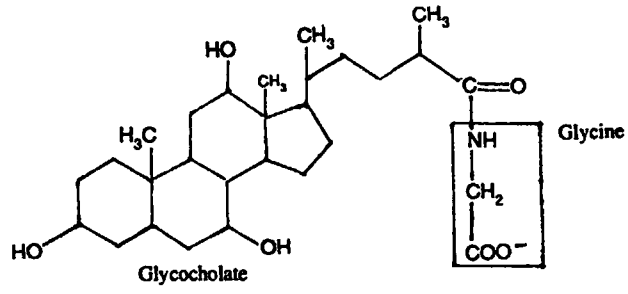


Bile Salts

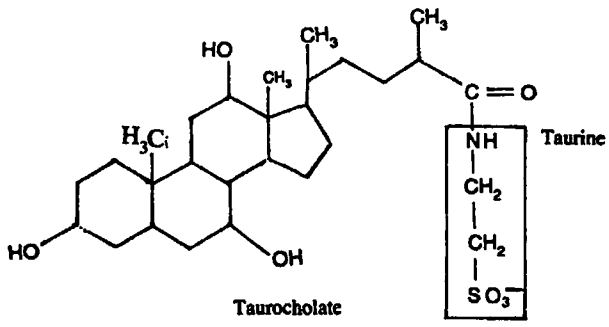
Bile salts are oxidation production of cholesterol. First the cholesterol is converted to the trihydroxy derivative, and the end of the aliphatic chain is oxidized to the carboxylic acid. The latter in turn forms an amide linkage with an amino acid, either glycine or taurine :

Bile salts are powerful detergents. One end of the molecule is strongly hydrophilic because of the negative charge, and the rest of the molecule is hydrophobic. Thus, bile salts can disperse dietary lipids and the small intestine into fine emulsions and thereby help digestion.

Since they are eliminated in the feces, bile salts also remove excess cholesterol in two ways : They themselves are breakdown products of cholesterol (thus cholesterol is eliminated *via* bile salts), and they solubilize deposited cholesterol in the form of the salt-cholesterol products.



Glycocholate

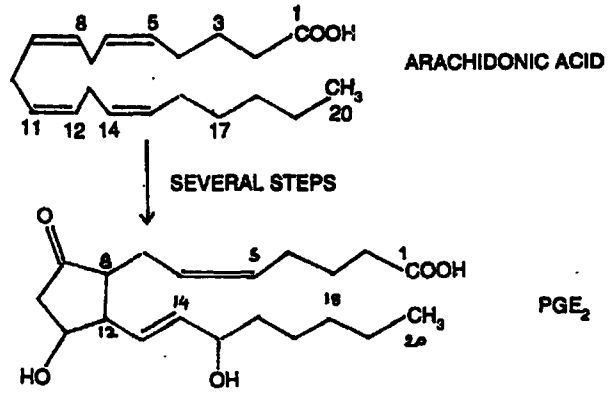


Taurocholate

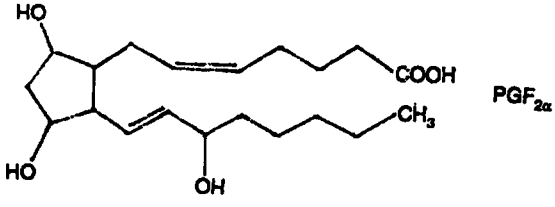
Prostaglandins

This group of fatty acid-like substances was first discovered when it was demonstrated that seminal fluid caused a hysterectomized uterus to contract. The name implies that these substances are a product of the prostate gland, and in mature males the seminal gland does secrete 0.1 mg of prostaglandin per day. However, small amounts of prostaglandins are present throughout the body in both sexes.

Prostaglandins are synthesized in the body from arachidonic acid by a ring closure at C-8 and C-12 :



The prostaglandin E group (PGE) has a carbonyl group at C-9, with the subscript indicating the number of double bonds. The prostaglandin F group (PGF) has two hydroxyl groups on the right at positions C-9 and C-11.

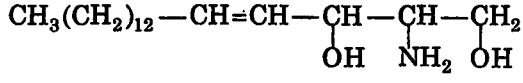


The prostaglandins as a group have a wide variety of effects on body chemistry. The seem to act as mediators of hormones, For example, PGE_2 and PGF_2 induce labour and are used for therapeutic abortion in early pregnancy. PGE_2 lowers blood pressure, but PGF_2 causes hypertension (increase of blood pressure). PGE_2 in aerosol from is used to treat asthma; it opens up the bronchial tubes by relaxing the surrounding muscles. PGE_1 is used as a decongestant; it opens up nasal passages by constricting blood vessels.

Many prostaglandins cause inflammation and fever. The analgesic effect of aspirin results from the inhibition of prostaglandin synthesis. PGA and PGE inhibit gastric secretions.

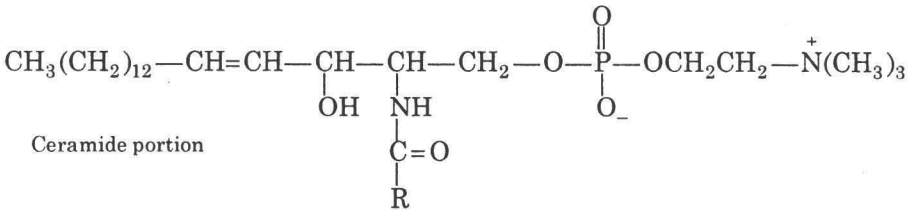
Sphingolipids

The coating of nerve axons (myelin) contains a different kind of complex lipid called sphingolipids. In sphingolipids the alcohol portion is sphingosine :

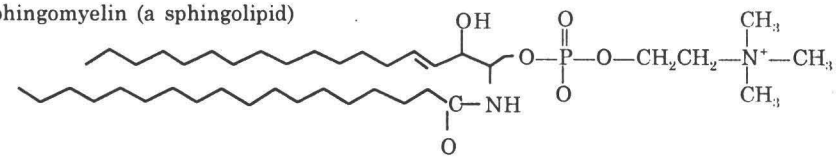


Sphingosine

A long chain fatty acid is connected to the $—NH_2$ group by an amide linkage, and the —OH group at the end of the chain is esterified by phosphorylcholine.



Sphingomyelin (a sphingolipid)



Sphingomyelin (schematic diagram)

The combination of a fatty acids and sphingosine is often referred to as the ceramide part of the molecule, since many of these compounds are also found in cerebrosides. The ceramide part of complex lipids may contain different fatty acids; stearic acid occurs mainly in sphingomyelin.

Cholesterol and Heart Attack

Like all lipids, cholesterol is insoluble in water, and if its level is elevated in the blood serum, plaque-like deposits may form on the inner surfaces of the arteries. This leads to a decrease in the diameter of the blood vessels, which may lead to a decrease in the flow of blood. *Atherosclerosis* is the result, along with accompanying high blood pressure, which may lead to heart attack, stroke, or kidney dysfunction. Atherosclerosis enhances the possible complete blockage of some arteries by a clot at the point where the arteries are constricted by plaque. Furthermore, blockage may deprive cells of oxygen, and these may cease to function. The death of heart muscles due to lack of oxygen is called *myocardial infarction.*

The more general condition, *arteriosclerosis*, or hardening of the arteries with age, is also accompanied by increased levels of cholesterol in the blood serum. While young adults may have, on the average, 1.6 g of cholesterol per litre of blood, above age 55 this almost doubles to 2.5 g/L because the rate of metabolism slows with age. Diets low in cholesterol and saturated fatty acids usually reduce the serum cholesterol level, and a number of drugs are available that inhibit the synthesis of cholesterol in the liver. Although there is a good correlation between high serum cholesterol and various circulatory diseases, not everyone who suffers from hardening of the arteries has high serum cholesterol, nor do all patients with high serum cholesterol develop arteriosclerosis.

15 Hormones

15.1 Introduction

A hormone is defined as a chemical compound produced in certain specialised cells, usually in a ductless gland, called *endocrine gland,* which is delivered directly to be blood stream in minute amounts and which exerts a psychological effect at a site of action remote from its origin. It is required in small quantities and specific in its functions. The deficiency of a hormone leads to a particular disease which can be cured by the administration of that hormone.

Although hormones have been found to stimulate some of the physiological functions, yet there are certain endocrine secretions which inhibit activity. Such endocrine secretions have been named as *chalones.*

The exact mechanisms by which hormones exert their effect are not known for any of the hormones. However, there is a definite evidence that some hormones exert their control by acting either directly or more likely on the DNA of the genes.

15.2 Differences Between Hormones and Vitamins

(*i*) Hormones are produced in the animal body while vitamins are synthesised in the plant body.

(*ii*) For hormones animals are independent in producing in the body whereas for vitamins, the animals depend upon the plants.

15.3 Differences between Hormones and Enzymes

The hormones differ from enzymes in the following ways :

1. The hormones are produced in an organ in which they ultimately perform their function.
2. They are secreted into the blood prior to use.
3. Structurally, they are not always proteins.

15.4 Properties of Hormones

These are as follows :

(*i*) They have low molecular weights. Due to this, they pass out through the capillaries.

(*ii*) They are produced in very low concentration. Therefore, they act like vitamins in low concentrations.

(*iii*) They are antigenic.

(*iv*) Whenever their function is over, they get readily destroyed or inactivated or excrete.

(*v*) They are probably acting as organic catalysts, *i.e.*, as coenzymes of other enzymes in the issue.

15.5 Hormones Secreting Glands

The main hormone secreting glands are intestinal mucosa, pancreas, adrenals, thyroids, parathyroids, pituitary, ovaries and testes. These glands have been grouped according to location in the following heads :

(*i*) *Cranial endocrine glands* : These are lying in the head. Examples are pineal and pituitary.

(*ii*) *Pharyngeal endocrine glands* : These are lying in the neighbourhood of the pharynx. Examples are thyroid and parathyroid glands.

(*iii*) *Abdominal glands* : These are lying in the abdomen. Examples are pancreas, intestinal glands and adrenals.

The summary of the various hormones produced and their functions have been given in Table 15.1.

Table 15.1
HORMONE PRODUCING ORGANS, HORMONES PRODUCED BY THEM AND THEIR FUNCTIONS

Organ	*Hormone*	*Function*
1	*2*	*3*
Adrenals	Epinephrine (adrenalin)	In glycogen breakdown
	Aldosterone	In mineral balance, Na retention
	Cortisol	In metabolism, gluconeogenesis
Gastro intestinal tract	Gastrin	In secretion of HCl by intestine
	Pancreozymin	In secretion of pancreatic juice
	Parotin	In calcification of teeth and change in Ca and P content of the serum
Pancreas	Insulin	In regulation of blood sugar
	Glucagon	In regulation of blood sugar
Hypothalalmus	Corticotropin releasing hormone	In release of corticotropin
	Thyrotropin releasing hormone	In release of thyrotropin
	Growth hormone releasing hormone	In release of somatotropin
	Gonadotropin releasing hormone	In release of luteinizing and follicle stimulating hormones

(Contd.)

1	*2*	*3*
Ovary-Follicles	Estradiol	In proliferation of the uterine mucosa and maintenance of the normal size and function of other female reproductive organs
—Corpus luteum	Progesterone	In converting the uterine mucosa to a secretory phase
Parathyroid	Parathormone	In maintenance of Ca and P level
Pituitary		
—Anterior lobe	Somatotropine	In growth and metabolism
	Carticotropine	In stimulation of the adrenal cortex
	Thyrotropine	In stimulation of thyroid gland
	Follicle stimulating hormone	In stimulation of maturing of gametes
	Luteinising hormone	In stimulation of production of sex hormones
—Middle lobe	Prolactin	In stimulation of mammary glands
—Posterior lobe	Melanotropin	In dialation of melanophores
	Vassopressin	In renal reabsorption of water
	Oxytocin	In contraction of uterus and expression of milk by lactating mammary gland
Testis	Testosterone	In normal development of male reproductive organs and secondary sex characteristics
Thyroid	Thyroxin	In increasing basal metabolic rate, development
	Calcitonin	In maintenance of Ca level in blood and bones.

15.6 Chemical Nature of Hormones

It is possible to classify hormones in accordance to their chemical structure or mode of action into the following types :

(i) *Steroid hormones :* The sex hormones and the hormones of adrenal cortex are steroids. They are fat soluble compounds. Examples of steroid hormones are aldosterone, cortisone, progesterone, etc.

(ii) *Amino acid derived hormones :* They include hormones such as thyroxine, epinephrine, nor-epinephrine, etc. Thyroxine is a thyroid hormone which is simply an amino acid (thyroxine).

(iii) *Peptide and protein hormones :* The hormones are water soluble and include oxytocin, insulin, secretin, relaxin, vasopressin, somatotropin, adrenocorticotrophic, etc.

(iv) *Glycoprotein hormones :* These include thyrotropin, follicular stimulating hormone and lutenizing hormones secreted in the adrenohypophysis of the pituitary.

(v) *Fatty acid derivatives :* These include prostaglandins.

15.7 Mechanism of Action of Hormones

Although the hormones circulate in the blood throughout the whole body, yet they act only on certain specific organs or tissues which are termed as *target organs* or *target tissues*. The cells in target tissues have a receptor protein which undergoes binding with the hormones. Hormones may give rise to desired physiological effects either in this form or after being transported to its site of action inside the cell with receptor protein.

At molecular level two types of reactions have been initiated by the hormones, see (1) and (2). However, the most recent and most convincing is that hormones work at the gene level [see (3)].

1. Synthesis of a new protein : There are many steroid hormones which induce synthesis of certain enzymes or new proteins in the target tissues. This induction does not take place in the presence of protein synthesis inhibitors like cycloheximide, puromycin and actinomycin D. Although hormone is known to induce any one of the steps in protein synthesis it is accepted that they have been found to act at transcription level. They combine with the repressor molecule and the inhibited DNA expresses itself in the form of mRNA molecules. These mRNA molecules will be translated to a new protein which may be an enzyme. The action of steroid sex hormones on the target cells gives rise to the synthesis of many new proteins. The mechanism of action of estrogen on oviduct cells has been investigated in detail. This has been briefed as follows :

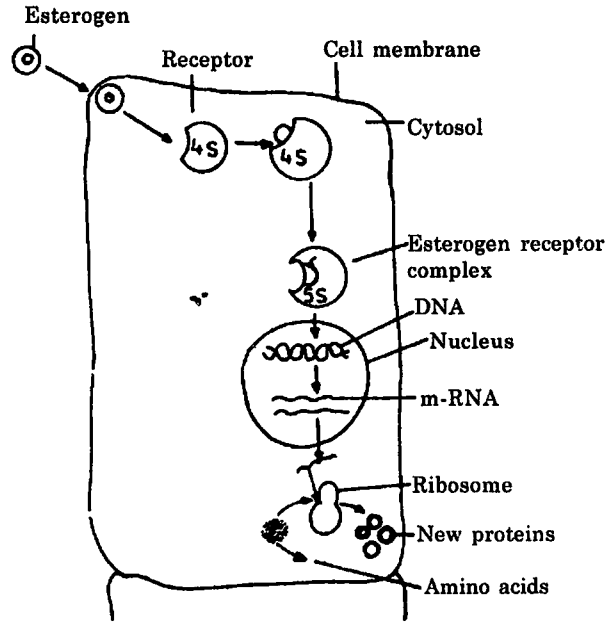


Fig. 15.1. Action of estrogen on target (oviduct) cells.

First of all, the hormone binds with a receptor protein present in the cytosol of the receptor cell (Fig. 15.1) to form a new protein which sediments at 4 S and is having molecular weight about 200,000. The protein occurs in only those cells which are responsive to estrogen. It undergoes binding covalently to the estrogen. After binding with the hormone, the protein gets changed into a form which is sedimenting at 5 S. The 5 S estrogen receptor complex now moves from cytoplasm to the nucleus. There it binds with the DNA and tends to increase the activity of DNA dependent RNA polymerase. This results in an overall increase in mRNA and now several new proteins have been synthesized.

2. Activation of enzyme and involvement of c-AMP : There are some enzymes which are activated in the presence of hormones. E.W. Sutherland and T.W. Rall (1960) reported that this activation gets mediated *via* a special form of the adenine nucleotide 3′—5′, cyclic AMP (c-AMP) called a second messenger while the hormone itself was the first messenger. The hormone after it reaches the receptor cell has been found to catalyse the conversion of ATP to 3′—5′, cyclic adenosine monophosphate and inorganic pyrophosphate. Cyclic AMP has been found to activate certain rate limiting enzymes by phosphorylating them. The activation of enzyme brings about

the change in the metabolic rate of the tissue and the desired physiological effect has been achieved. The events giving rise to the desired effect of a hormone through c-AMP have been given in Fig. 15.2.

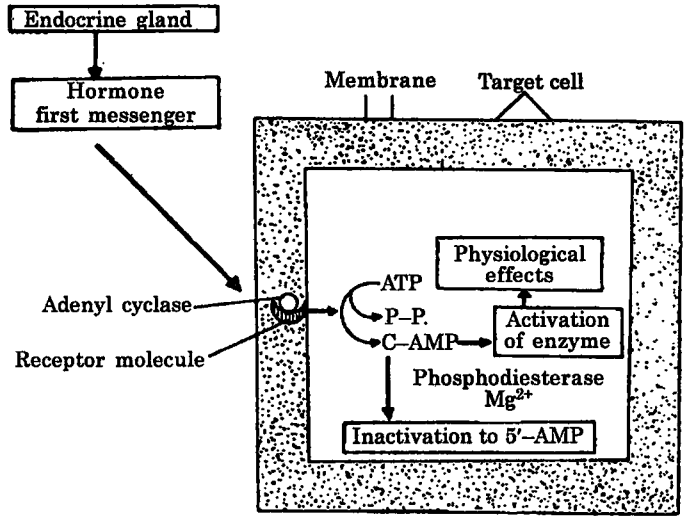


Fig. 15.2. Role of Cyclic AMP in hormone action.

Several hormones such as adrenalin, glucagon, vassopressin and parathormone are known which have been found to be mediated through an increase in c-AMP. However, the activation of enzyme by c-AMP does not take place in all cases. It has been reported that cyclic AMP affects the permeability of membrane (probably through the regulation of calcium level in the membrane) and the synthesis of certain proteins which are involved in the transport. It has been reported that the effects of aldosterone on movement of Na^+ through the kidney and increased uptake of glucose by the target organs of thyrotropin and luteinizing hormones have been found to be due to the effects of hormones on membrane permeability.

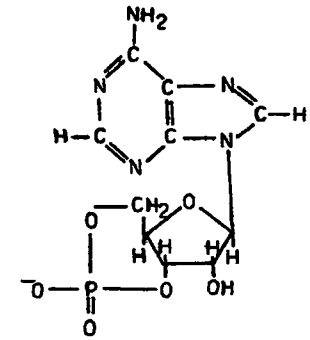


Fig. 15.3. Cyclic (3' 5') AMP.

3. Working of hormone at the gene level : The most recent and most convincing concept is that hormones work at the *gene level.* The gene level theory originated in work in Germany on the insect hormone *ecdysone.* It has been reported that ecdysone activates certain genetic loci on the chromosomes of the salivary glands of diptera and this activation can be shown by puffing of specific regions. Some workers investigating bacteria have demonstrated that the antibiotic actinomycin blocks the formation of new RNA.

It has been proved that hormones initiate the transcription of specific parts of the genetic code, or, initiate the formation of RNA presumably by combining with genetic repressors. New RNA, of course, means new enzymes. New enzymes mean changes in metabolism and a wide range of possible new products.

Clear evidence comes from studies of female sex hormone. If the ovaries of a rat are removed, the endometrium lining the uterus remains permanently in the reduced, anestrus, condition. If estrogen is then injected into the rat, an increase in uterine RNAs can be detected within half an hour. After three or four hours an increase in protein can be measured and the uterus begins to grow into the estrus condition. These facts in themselves support the theory.

15.8 Some Important Hormones

Sex hormones : The sex hormones are a group of steroids biosynthesised in the testes, ovaries, adrenals and placenta of pregnancy. The activity of these sex hormones is controlled by the hormones that are produced in the anterior lobe of the pituitary gland.

The sex hormones regulate reproductive functions and are responsible for the development of secondary male and female sex characteristics.

Since the initial isolation and identification of the sex hormones, important structurally modified steroids with high biological activity and specially desirable therapeutic properties have become available for administration either orally or by injection.

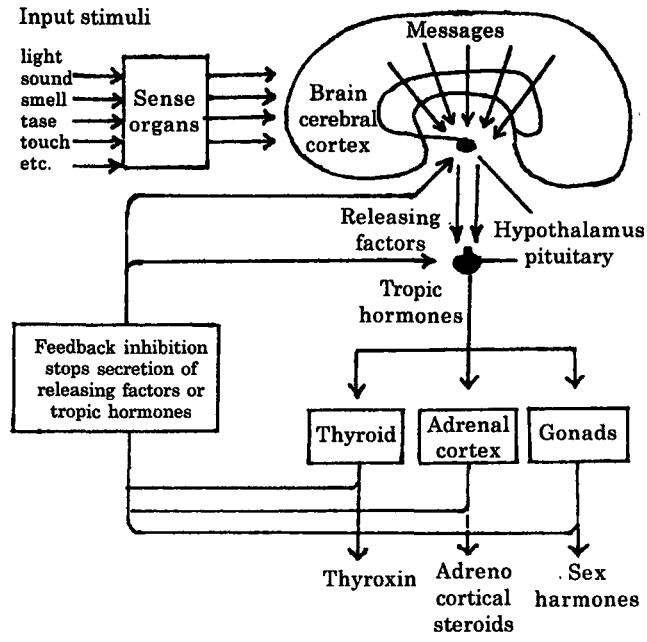


Fig. 15.4. Regulation of hormonal secretion.

The sex hormones are of three types :

(a) Androgens : These are male hormones.

(b) Oestrogens : These are female or follicular hormones.

(c) Gestogens : These are corpus luteum hormones.

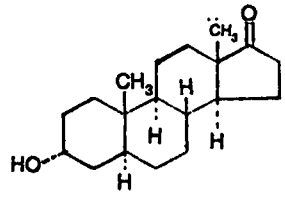
Sex hormones are responsible for the sexual processes, and for the secondary characteristics which make differentiation between males and females.

Androgens

(a) Androsterone : Of the male sex hormones which approach the activity of testosterone, androseterone is the most potent, having approximately 1/7 of the activity of testosterone.

Buternandt (1913) isolated the first male hormone, namely, androsterone. He obtained 15 mg of the crystalline androsterone from 15,000 litres of urine. It has been established that urine contains approximately 1 mg of androsterone per litre of urine.

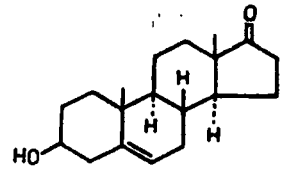
The androsterone occurs in urine as a conjugate with glucuronic acid. This also occurs in blood as sulphate. This has also been traced in small amounts in adrenal cortex, human sperm and ovary.



Androsterone

(b) Dehydroepiandrosterone : Butenandt (1931) isolated the first male hormone, namely, androsterone from urine. The activity of this hormone is from 1/3 to 1/2 that of androsterone. It

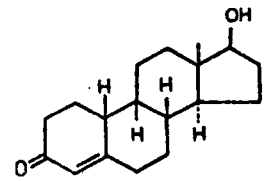
is of interest to note that dehydroepiandrosterone has been obtained from the urine of pregnant and non-pregnant women. Its structure resembles quite markedly the structure of oestrone.



Dehydroepiandrosterone

(c) Testosterone : There are at least 5 steroidal hormones which exhibit a typical androgenic activity. All are derivatives of androstane. However, the most potent so far is testosterone. Of the other compounds which approach the activity of testosterone, androsterone is the most potent, having approximately 1/7th of the activity of testosterone.

Testosterone was isolated for the first time by E. Laqueur *et. al.* (1935) from testes. From 100 kg of testes they obtained 10 mg of testosterone. The earlier attempts of isolating testosterone failed due to its instability in the presence of alkali.



Testosterone

Testosterone appears to be the real male sex hormone, others are metabolic products of it. The various function of testosterone are as follows :

(*i*) Testosterone promotes growth of secondary sex organs—epididymis, vas deferens, prostate, seminal vesicles and penis. It also promotes muscular and skeletal growth and is protein anabolic.

(*ii*) This assists in bringing about the descent of the testis in cryptorchidism.

(*iii*) This inhibits the secretion of the anterior pituitary gonadotropins (Moore, 1935).

(*iv*) Testosterone and its derivatives have been found useful in the treatment of advanced metastatic carcinoma of the breast.

(*v*) Occasionally, testosterone may produce jaundice.

(*vi*) Testosterone is used in the treatment of the menopausal syndrome, combined with oestrogens.

(*vii*) It exerts protein anabolic effect by increasing the RNA and RNA polymers of the cell nucleus and aminoacyl transferase of the ribosome.

Estrogens : Estrogens are a class of organic compounds which, in general, reverse most of the effects of ovariectomy and specially stimulate the female secondary sex characters such as the vagina, uterus, mammary glands, and fallopian tubes. Other biological activities of oestrogens include inhibitory effects on the synthesis and release of gonadotropic hormones of the anterior pituitary by way of the hypothalamic area of the brain.

Estrogens can be isolated from urine, placentas and reproductive organs of mammals. For example, in an original extraction, about 70 units of activity, (*i.e.*, about 0.07 mg of oesterone) were obtained from 1 litre of urine received from pregnant women.

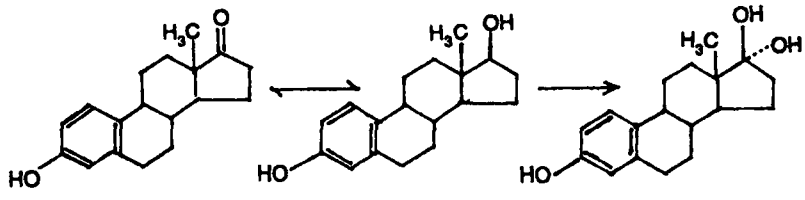
In woman, the hormones are concerned in the preparative phase of the menstrual cycle. They induce proliferation of endometrium, deepening or uterine glands, increased vascularity and changes in the fallopian tubules and vagina.

They suppress the secretion of FSH of pituitary. Estrogens are necessary for the maintenance of female secondary sex characters.

The hormones also act as cofactors in the transhydrogenation between the two pyridine nucleotides NADP and NAD.

$$NADPH + NAD \xrightarrow[\text{Transhydrogenase}]{} NADP + NADH$$

Metabolic Action of Estrogens : They exert protein anabolic effect locally on the target organ (uterus). The hormones are bound to a specific lipoprotein in that tissue. The RNA polymerase activity of the tissue is increased. They also increase the phospholipid turnover rate in general and are hence lipotropic. They bring down the plasma lipids, if administered in conditions of hyperlipemia and coronary heart disease in men. Serum calcium and phosphate levels are increased on prolonged administration. Hypercalcification of bones may occur.

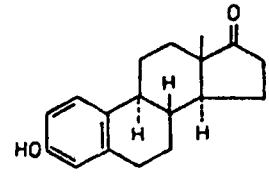


Estrone Estradiol Estriol

Some members of estrogens are estrone, estriol, estradiol and equilenin group of estrogen.

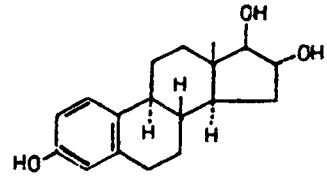
(a) Estrone : It is the first known member of sex hormones. If was isolated by Butenandt and Doisy independently in 1929 from the urine of pregnant women.

It is a stable white substance existing in three crystal forms : rhombic metastable (m.p. 254°C), monoclinic metastable (m.p. 256°C) and rhombic stable (m.p. 259°C); [α] + 170° (dioxane); maximum absorption in the ultraviolet (in alcohol), 280 nm.



(±) Estrone

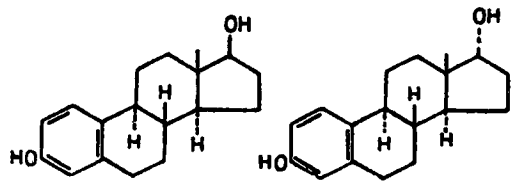
(b) Estriol : It was isolated from human pregnancy urine by Marrian (1950). It is useful in some cases of estrogen deficiency, especially for the relief of menopausal symptoms and, in pediatrics, to combat gonorrheal vaginitis. Estriol is effective when taken orally. It occurs mainly in human placenta and pregnant women.



Estriol

(c) Estradiol : There are two stereoisomeric estradiols, α- and β-. Out of these β-isomer is much more active than estrone whereas α-isomer less active.

The β isomer was isolated from the ovaries of sows (Doisy *et al.*, 1935). The α-isomer was isolated from the pregnancy urine of mares (Wintersteiner *et al.*, 1938). The α-isomer has m.p. of 178°C whereas the β-isomer has m.p. of 222°C.

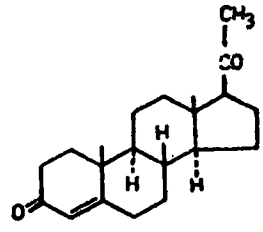


Estradiol-17β Estradiol-17α

Gestogens or Progestation Hormones or Luteal Hormones

(a) Progesterone : It is one of the most important members of gestogens.

It was early suspected that corpus luteum was a gland with inner secretions and in 1928 Corner and Allen made the important observation that progestational changes which had been prevented by removal of the early corpus luteum could be restored by the use of corpus luteum extracts. In 1932, a crude, crystalline material that had physiological activity was isolated. In 1934, the isolation of the pure progestational hormone, progesterone, was announced independently by four laboratories.



Progesterone

Progesterone has been isolated from the corpus luteum, the adrenal cortex, the placenta and the testis. During the period of full corpus luteum activity the human ovary produces about 1000 mg of progesterone daily.

Progesterone is a C-21 compound and is synthesized from the common precursor, pregnenolone.

The hormone is also formed by the placenta and also by adrenal cortex where it is the precursor of the several cortical hormones.

The action of the hormone is to cause endometrical development preparatory for the reception and nutrition of the embryo. It suppresses ovulation, estrus and the secretion of LH by pituitary. During pregnancy, progesterone production by corpus luteum continues through, till near term.

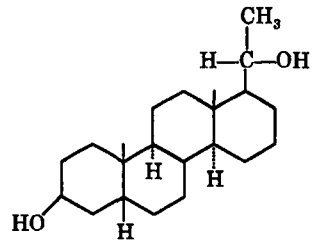
If pregnancy has not occurred, the output of both estrogen and progesterone suddenly fall on or about the twenty eighth day of the menstrual cycle, menstrual flow starts and the uterine endometrium starts sloughing.

Three-fourths of the progesterone is eliminated through bile through feces as pregnanediol and pregnanetriol and also through urine.

Progesterone exerts an anti-ovulatory effect, if administered from 5th to 25th day of the menstrual cycle. It is however active only on parenteral administration. Synthetic progestins like 17 α- ethinyltestosterone and 17 α- ethinyl, 19-nortestosterone are active on oral administration, their activity being equal to or more potent than that of parenteral progesterone.

Relaxin is another hormone from the corpus luteum. It causes relaxation of symphisis pubis in animals. It is a polypetide hormone and is also produced by the placenta.

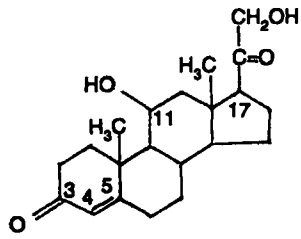
(b) Preganaediol : It is biologically inactive metabolic product of progesterone. First of all, it was isolated from human pregnancy urine by Marrian (1919).



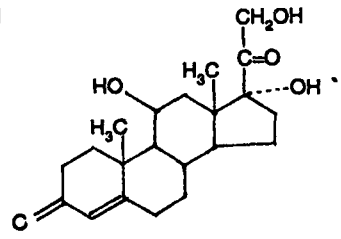
Pregnanediol

Hormones Secreted by Adrenal Cortex

Adrenal cortex secretes several hormones all of which are steroid hormones. Kendall and associates isolated and studied a number of the hormones. They exert profound effects on (*i*) mineral metabolism, (*ii*) carbohydrate, fat and protein metabolisms (mainly carbohydrate) and (*iii*) sex hormones like actins. Nearly 50 distinct steroidal compounds are isolated from adrenal cortex and they are classified according to their principal actions into one of three groups. (*a*) mineralocorticoids, (*b*) glucoroticoids and (*c*) sex hormones. The sex hormones may be estrogens (18 carbon compounds), androgens (with 19 carbons) and progesterones (with 21 carbons). They resemble similar hormones produced by ovary and testis and are not further considered here. The mineralocorticoids and glucocorticoids have 21 carbons each.

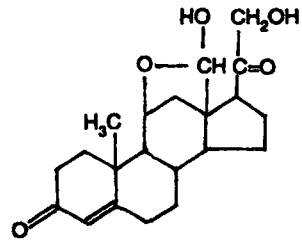


Corticosterone
(Compound-B)

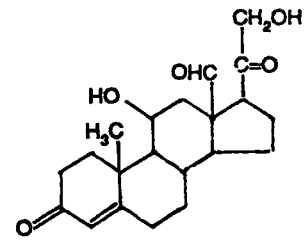


17-Hydroxy corticosterone
(Hydrocortisone, cortisol
or compound-F)

Chemistry : The parent ring structure is the 'pregnane' ring. The active hormones have a double bond between C-4 and C-5, and two keto groups-one at position 3 and another at position 20. Certain key groups exert profound influence on the biological activity of the compounds. A 'OH' at position 21 enhances sodium retention and is necessary for the effect on carbohydrate metabolism. A hydroxy or ketonic group at position 11 is also necessary for the effect on carbohydrate metabolism but it diminishes the effect on mineral metabolism. A hydroxy group at position 17 enhances activity on carbohydrate metabolism.



Aldosterone
(hemiacetal form)



Aldosterone
(Aldehyde form)

Fig. 15.5. Adrenal Cortical Hormones.

The structures of the more important hormones are shown in Fig. 15.5.

If in position 11 there is an 'O' instead of 'OH' it is 11 dehydrocorticosterone (compound A). If there is neither 'O' nor 'OH' it is 11 deoxycorticosterone.

Similarly 11-dehydro 17-hydroxy corticosterone (compound E or cortisone) and 11-deoxy, 17-hydroxy corticosterone (compound S). Replacement of the CH_3 at position 13 by a CHO (aldehyde group) gives rise to aldosterone, the potent minerals corticoid. It can exist in the aldehyde form or the hemiacetal form.

The mechanism of action of the adrenal cortical hormones is at the level of the cell nucleus. They bind to specific receptor proteins in the cytosol and the steroid receptor complexes enter the nucleus where they bind to specific sites on the chromatin transiently and influence mRNA synthesis and through that protein (enzyme) synthesis. Inhibitors of RNA synthesis will thus prevent action of these hormones.

Functions

1. Mineral metabolism : They increase the reabsorption of sodium and chloride by the renal tubule and decrease their excretion in sweat, saliva, and gastro-intestinal secretions. Aldosterone is the most potent hormone in this regard. 11-Deoxy corticosterone and 11-deoxy, 17 hydroxy corticosterone (compound S) also have profound effects. They are produced in the zona glomerulosa. 11-Deoxycorticosterone acetate (DOCA) can be synthesized in the laboratory and can be absorbed by the buccal mucosa. Hence it can be administered sublingually as a substitute for aldosterone, though it is only 4% as active as aldosterone.

There is increased excretion of potassium (exchanged for the sodium being reabsorbed) and increased retention of sodium and water in the body.

Aldosterone secretion is not altered by the adrenocorticotropic hormone (ACTH). Low sodium intake in food causes an increase in production of aldosterone and vice versa. The regulatory mechanism seems to act through alteration of extracellular fluid volume, decrease of which stimulates aldosterone secretion which facilitates retention of sodium and an equivalent amount of water.

A specific aldosterone stimulating hormone (A.S.H.) is also said to function. Volume receptors said to be present in the walls of the renal afferent arterioles are stimulated by a decrease in the stretch of the arteriolar wall (fall in E.C.F. volume. This causes secretion of renin by the juxatglomerular cells which in turn causes secretion of 'angiotensin II.' The angiotensin II stimulates the relevant cells of the zona glomerulosa (of the adrenal cortex) to secrete aldosterone. Aldosterone secretion will lead to retention of sodium and water and expansion of ECF volume and thus causes stretching of the efferent arterioles of glomeruli and cuts down in the secretion of renin. This is a 'feed back' mechanism of regulation.

Increased potassium concentration of plasma directly stimulates aldosterone production independent of ECF volume. The hormone seems to act at the nuclear level on the tubular epithelial cells.

2. Carbohydrate, lipid and protein metabolism : Hormones with a 'O' or 'OH' at position 11 exert their effects mainly on carbohydrate metabolism and are called the glucocorticoids or the 'S' hormones. Corticosterone (compound-B), 11-dehydrocorticosterone (compound-A) and the 17-hydroxy derivatives of these (compounds E and F) belong to this group. They are produced mainly in the zona fasciculata and zona reticluaris. They are insulin antagonists in many respects. They cause (1) increase in blood sugar level; (2) decrease the utilization of carbohydrate; (3) increase the synthesis of glycogen; (4) increase gluconeogenesis; (5) decrease lipogenesis and increase lipolysis; (6) decrease reabsorption of uric acid by renal tubules and thus produce an increased excretion of uric acid and in urine (uricosuric effect); (7) cause involution of thymus and eosinopenia and lymphopenia and (8) increase gastric HCl and pepsin production by stomach

and trypsin production by pancreas. (9) Stress : Glucocorticoids help to raise the blood pressure which falls in emotional or surgical shock.

The mechanisms of action for these diverse effects of the hormone are not clear. Their stimulation of gluconeogenesis seems to be on account of stimulation of the enzyme pyruvate carboxylase. This and other enzymes concerned in gluconeogenesis and amino acid catabolism seem to be synthesized in larger amounts due to the stimulant action of the hormone on the cellular RNA synthesis in the liver.

Anti-inflammatory effects : Besides the above metabolic effects, the cortical hormones exert a profound anti-inflammatory effect, and are invaluable in the treatment of collagen diseases like rheumatoid arthritis. They also decrease the antigen-antibody response and are useful in treating allergic conditions. They probably suppress synthesis of nucleic acid and protein in the lymphocytes. Cortisol also depresses immune response in organ transplantation procedures.

Regulation of secretion : ACTH regulates the secretion of glucocorticoids. Lowered cortisone levels in blood stimulate ACTH production.

Biosynthesis of the hormones : The steps in synthesis are similar to cholesterol synthesis and start with the simple 2-carbon molecule–acetyl coenzyme-A. The synthesis requires NADPH + H^+. Adrenal cortex is rich in ascorbic acid which has also probably a role in some of the reduction steps.

Pregnenolone is one of the important precursors formed. This can now follow the androgen pathway or the corticosteroid pathway. Progesterone is the first hormone formed from pregnenolone. 'Hydroxylases' capable of introducing an OH group at 11, 17 or 21 positions are required in the pathways. Abnormalities in these enzymes are responsible for several defects in adrenal cortical function.

About 25 mg of cortisol and 2.5 mg of corticosterone and less than 0.1 mg of aldosterone are the principal hormones secreted by the adrenal cortex per day.

Fate of adrenal cortical hormones : The hormones are transported bound to an α-globulin in the plasma (transcortin, corticosterone-binding-globulin; CBG). In the liver, they are reduced to their tetrahydro derivatives and then conjugated with glucuronic acid and excreted through bile. Some are reabsorbed (entero-hepatic circulation) and a portion enters systemic circulation to be excreted by the kidney. Thus liver is an important organ in inactivating the hormones. In hepatic failure, one cause for edema and sodium retention may be on account of prolonged action of these hormones due to failure of the liver to inactivate them.

Non-Steroid hormones

Examples of non-steroid hormones are as follows :

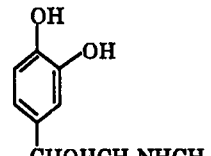
(*a*) Adrenaline (Epinephrine)

(*b*) Nor-adrenaline (Nor-epinephrine)

(*c*) Thyroxine.

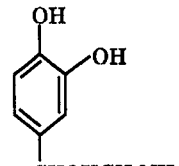
(a) Adrenaline : The *adrenal medulla* secretes two hormones, *adrenaline* and *nor-adrenaline*. The two are secreted in the ratio of 4 : 1. The former is twice *active* than the latter. Adrenaline is also found in poisonous secretions of some toads. It was the first hormone to be extracted from the animal tissue and synthesized.

It raises the blood pressure and is used locally to stop haemorrhage. However, it is active only when it is given by injection. It increases blood pressure by releasing glucose from the stores of glycogen of liver.



Adrenaline
(Epinephrine)

(b) Nor-adrenaline : It is secreted by the adrenal medulla and released at the sympathetic nerve endings, producing a variety of physiological effects, including a rise in blood pressure and blood sugar, dilation of the pupil of the eye, acceleration of the heart rate, relaxation of the bronchial muscles and constriction of some blood vessels. The natural compound is laevorotatory whereas the synthetic compound is a racemic mixture.



Nor-Adrenaline
(Norreepinephrine)

Functions of norepinephrine and epinephrine : The general physiological effects are briefly summarized below :

1. Norepinephrine exerts an overall vasoconstrictor effect without much effect on cardiac action. The blood pressure is increased.

Epinephrine causes vasodilatation of arterioles of muscle and vasoconstriction of arteioles of skin and splanchnic area besides increasing rate and force of contraction of the heart. The overall effect is a rise in blood pressure.

2. Epinephrine causes relaxation of smooth muscles of the stomach, intestine, bronchioles and urinary bladder and contraction of the sphincters of stomach and bladder. Its relaxant effect on bronchioles is used in the treatment of bronchial asthma.

3. *Metabolic effects* : Epinephrine is by far more potent than norepinephrine in metabolic functions :

(*a*) *Liver glycogenlysis* : The action of epinephrine is similar to that of glucagon. By stimulation of the liver enzyme, adenyl cyclase, there is a final increase of phosphorylase activity and glycogenolysis, causing an elevation of blood sugar level.

(*b*) *Muscle glycogenolysis* : Unlike glucagon, epinephrine enhances the cyclic AMP level in muscle also and cause glycogenolysis in it leading to increased blood pyruvate and lactate levels.

(*c*) Lipolysis is stimulated in adipose tissue leading to a rise in the non-esterified fatty acid (NEFA) levels of plasma.

4. Glucose uptake by tissues is diminished.

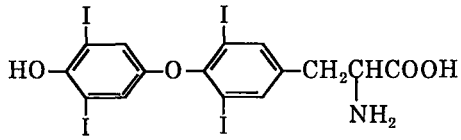
5. Insulin secretion by pancreas is decreased.

The above action results in (1) increased output of glucose by liver glycogenolysis. (2) Increased gluconeogenesis by liver from pyruvate and lactate and fatty acid (made available by epinephrine action on muscle and adipose tissue) also helping in increased output of glucose by liver and

(3) diminished uptake of glucose by peripheral tissues. The glucose thus passing out into circulation from liver is readily and exclusively available to the nervous system to help in tiding over and emergency or stress.

(c) Thyroxine : It is a hormone which is secreted by thyroid gland in the form of protein called *thyroglobulin.* The latter compound when hydrolyzed yields mainly thyroxine, common amino acids and various iodinated derivatives—L-histidine (4—) L-tyrosine (3- and 3,5.) and L thyronine. Three iodothyronines, which are present, are 3, 3′; 3, 3′, 5′—; 3, 3′, 5—. Out of these, only the last compound exhibits biological activity.

Thyroxine is an iodine derivative which was first isolated by Kendall (1919) and later isolated by Harington (1930) as a white crystalline solid, m.p. 235°C, $[\alpha]_D$ 4.4°.



(±)-Thyroxine

Biosynthesis : The thyroid has the ability to take up iodine from plasma against a concentration gradient and utilizes it for hormone synthesis. About 1/3 of the inorganic iodine of plasma derived from food is taken up by the gland, and the rest is excreted mainly by kidney. Small amounts are also excreted in saliva, milk and gastrointestinal secretions. The iodine concentration in thyroid is ten to hundred times that in plasma. The TSH (thyroid stimulating hormone) of the pituitary stimulates the uptake of iodine by the gland. Thiocyanatee and perchlorate competitively inhibit iodine uptake. Cyanide and dinitrophenol also inhibit the uptake by blocking cellular metabolism.

In the gland, the inorganic iodide (I^-) is oxidized to iodine (I or I^+) by a peroxidase enzyme with the loss of one or two electrons. The active iodine is now taken up by the tyrosine moiety of the glycoprotein, thyroglobulin, which is the characteristic protein of the thyroid and has a molecular weight of 660,000. It has 115 tyrosine residues in its molecule. The sequence of iodination of tyrosine and the condensation of iodotyrosines to form the hormones are shown in Fig. 15.6.

The iodination of tyrosine and the subsequent coupling reaction between the iodotyrosines all occur while in the thyroglobulin molecule. The tri-iodo and tetraiodothyronine (T_3 and T_4) are then released by proteolytic enzymes present in lysosomes or vesicular membranes and enter the blood stream. Mono and diiodo tyrosine are also released, but are deiodinated and the iodine is again used. Release of the hormone from the thyroglobulin is accentuated by T.S.H. and by exposure to cold environment.

Functions of Thyroxine and Other Related Thyroid Hormones

Functions of Thyroid Hormones

1. Calorigenic effect : They increase the metabolism and oxygen consumption of all tissues.

2. Protein metabolism : In physiological levels, they are protein anabolic and are necessary for normal growth. In higher amounts, they cause excessive breakdown of protein due to an increased rate of metabolism and cause a negative nitrogen balance. There is increased excretion of urinary creatine.

3. Carbohydrate metabolism : The rate of absorption of glucose from the intestines is increased. This results in rapid hyperglycemia during an oral glucose tolerance test. But, since the rate of

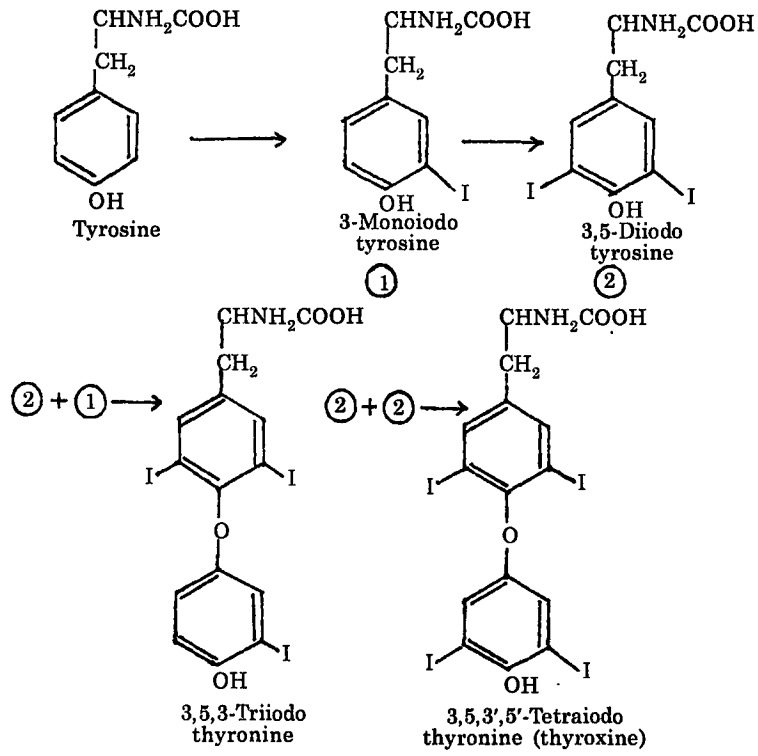


Fig. 15.6. Thyroid hormones.

metabolism of glucose of tissues is also high, the level rapidly comes down to normal. There is also an increase in hepatic glucose-6-phosphatase activity and a more rapid insulin destruction. Glycogenolysis is increased in the liver and muscle due to increased sensitiveness to epinephrine. Gluconeogenesis is also enhanced. All these effects lead to the development of a diabetic state. But the utilization of glucose by tissues through glycolysis as well as citric acid cycle is not impaired. On the other hand it is increased. Hence the diabetes is not of a severe type.

4. Lipid metabolism : The hormones favour lipolysis in the adipose tissue and raise the plasma NEFA levels. This may be also an indirect effect through sensitization of the tissue to epinephrine action. The lipogenic effect of insulin is also enhanced. The important diagnostic effect is on plasma cholesterol levels. In hyperfunction of the thyroid, there is an increased hepatic synthesis of cholesterol, but a greater increase in its oxidation to bile acids and elimination. The result is a decrease in plasma cholesterol levels.

In hypothyroidism, the reverse set of conditions act—a decreased synthesis but a greater decrease in oxidation leading to a rise in plasma cholesterol.

Mechanism of Action

The action of thyroid hormones is relatively slow. They enter the target cell and are bound to a specific carrier molecule which is directly associated with nuclear chromatin. In moderate concentrations they have an anabolic effect RNA content, amino acid transport into the cell and protein synthesis in the cell increase.

Higher concentrations of the hormones produce negative nitrogen balance. Protein synthesis is decreased; carbohydrate and lipid breakdown are increased; bone gets decalcified, mitochondrial swelling and uncoupling of oxidative phosphorylation occur.

Antithyroidal Substances

1. Thyroid hormone itself suppresses further secretion by the gland by a feedback mechanism. This action is mediated through T.S.H.

2. Deficiency of iodine in food and drink causes hyperplasia of thyroid (goitre). Excess of iodine causes hypofunction by decreasing T.S.H. output and is used in the treatment of hyperthyroidism.

3. Thiocyanate inhibits the uptake of iodine by the thyroid by blocking the concentrating mechanism.

4. Thiourea, thiouracil and sulfonamides inhibit synthesis of the thyroid hormone and produce goitre. The action may be preventing oxidation of iodide directly by their action on iodide or though inhibition of oxidizing enzymes.

Hypothyroidism : In children, hypothyroidism manifests as cretinism. In adults it produces myxedema.

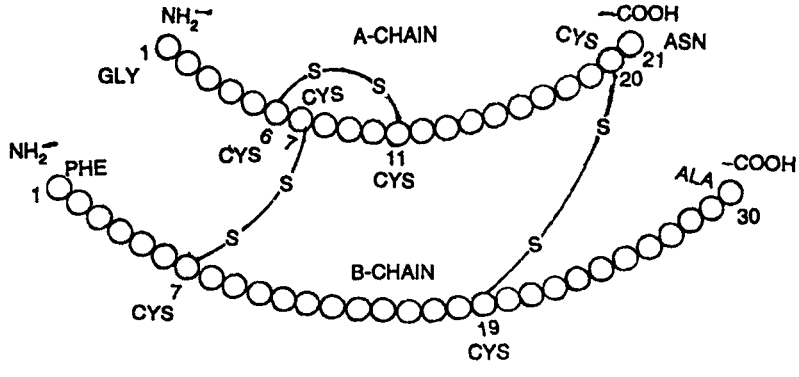
A cretin is dwarfish in size, has a thick tongue and thick skin, mentally retarded and sexually underdeveloped.

In myxedema, the skin is thick and puffy due to deposition of what is known as myxomatous material in subcutaneous layers: mental powers are dullened and there is hypersensitivity to cold.

The plasma cholesterol levels are elevated and B.M.R. decreased (—20 to —40%). Administration of thyroine will remedy the condition. Each milligram of thyroxine can raise the B.M.R. by 2.8 %.

Insulin

Von Mering and Minkowski demonstrated relationship of pancreatic hypofunction and diabetes mellitus as early as 1890. Banting and Best finally succeeded in extracting insulin from pancreas in 1922.



Chemistry : It is a protein hormone and can be purified and separated in a crystalline form. Traces of zinc are invariably associated with insulin. The protein structure has been elucidated by the brilliant work of Sanger and Smith. Human insulin is made up of two polypeptide chains (A & B) linked together by two disulfide linkages and has a total of 51 amino acids. It has a minimum molecular weight of 5734. It more often is present as an aggregate of two or more such simple molecules and shows a molecular weight from 12,000 to 48,000. Insulins from different sources (*e.g.*, pig, cattle, sheep, and horse) show minor differences in the amino acid composition and immunological activity. The nearest to human insulin in structure is insulin from pig. Insulin is destroyed by the action of digestive enzymes and is hence inactive when administered by mouth.

It is active when administered by injection. The biological action of the hormone can be prolonged by combining it with protamine or globin (protaminezinc-insulin and globin-insulin) or by altering the size of the crystals (ultralente insulin : large crystals and slow acting) so that it is slowly released from the site of injection and acts for several hours (12 to 24 hours). Lente insulin is a 7 : 3 mixture of ultralente and regular insulins.

Biosynthesis of insulin : The ribosomes of the endoplasmic reticulum of the β-cell are concerned in the synthesis of the hormone. A pro-insulin is first synthesized which is made up of the A and B chains linked together by another polypeptide containing 33 amino acids. The pro-insulin in the pig has a molecular weight of 9082. It undergoes a proteolytic cleavage and the insulin molecule separates out and forms aggregates of two to eight molecules. During the proinsulin stage, the β-cell is filled with vesicles containing the pro-hormone. Fully formed insulin is associated with the granules of the beta cell which form within the vesicles. In islet cell tumours, proinsulin itself may be circulating in the plasma.

Treatment in vitro of proinsulin with trypsin will remove the connecting polypeptide and release free insulin. In the process, alanine, the 30th amino acid of the B chain is also removed. It is called "dalanated insulin" (for dealaninated insulin) and has the same biological activity as insulin.

In recent years, a highly purified insulin-monocomponent insulin is being marketed. It has minimum antigenicity and is particularly useful in cases of insulin resistance.

The human pancreas is said to contain about 250 units of insulin at any one time. The daily requirement of the hormone is only about 50 units. 1 mg. of pure crystalline insulin has an activity of 24 units. Increase in the glucose concentration of the blood is a direct stimulus to insulin secretion. The granules move to the periphery of the β-cell and are extruded into the blood by a process called 'emeiocytosis' which is reverse process of pinocytosis. Simultaneous with release of insulin the polypeptide linking the A and B chains—the *c*-peptide—is also released into circulation. Glucose cannot only stimulate the release of the performed hormone, but can also stimulate further synthesis of the hormone by the beta cell. Sugars like mannose and fructose also act like glucose in stimulating insulin release.

It is likely that actual stimulus is provided not by the sugars themselves but by one of the products of their metabolism, probably arising out of the citric acid cycle. Cyclic AMP is also a stimulant for insulin secretion. Glucose stimulates calcium uptake and production of cyclic AMP by the beta cell. Localization of calcium in certain areas of the beta cell may be necessary for the secretion of insulin.

The role of fatty acids in stimulating insulin production is not clear.

Many of the hormones like growth hormone and glucocorticoids stimulate insulin secretion through their hyperglycemia effect. Glucagon seems to stimulate insulin production not only by causing hyperglycemia but also by increasing the cyclic AMP in the pancreas. The cyclic AMP stimulates glucose metabolism and thus supplies citric acid cycle intermediates which stimulate insulin secretion.

Epinephrine inhibits insulin secretion in spite of the hyperglycemia.

In vitro, calcium and potassium stimulate insulin secretion while magnesium is inhibitory.

Glucose taken by mouth is a better stimulus for insulin secretion than when taken by injection. This is attributed to the action of the intestinal hormones like pancreozymin, secretin and glucagon-like substances which are released when glucose is administered by mouth. They seem to stimulate insulin secretion.

Vagal stimulation increases insulin secretion.

Metabolism of insulin : Insulin is believed to be transported in the plasma bound to a specific insulin transporting protein. Insulin is degraded primarily in the liver and kidney by the enzyme "glutathione-insulin transhydrogenase" which cleaves the-S-S. linkages to SH, thus separating the A and B chains. The hydrogen is derived from glutathione. The A and B chains undergo further hydrolysis by the enzyme *insulinase*. The half-life of plasma insulin is only 7 to 15 minutes.

Mode of action of insulin : Muscle, adipose tissue and liver are the major sites of its action. It is also active on the lens and leukocytes. It has little action on the metabolism of the renal tissue, erythrocytes and the gastrointestinal tract.

1. Extrahepatic tissues : Insulin gets absorbed on to the cell membrane and probably is linked by an opening and reformation of one of the disulfide linkages in the molecule with the membrane protein. It facilitates the transport of glucose across the cell membrane. This being the rate limiting step in glucose metabolism, insulin promotes all subsequent metabolic pathways—glycogenesis, glycolysis and HMP-pathway. This results in production of more acetylcoenzyme A through glycolysis and more NADPH + H^+ from HMP pathway which in turn favours fatty acid synthesis and lipogenesis in the adipose tissue.

Insulin stimulates intracellular transport of all sugars which have same configuration as glucose in carbons 1, 2 and 3 (*e.g.*, arabinose, xylose and galactose). Fructose (which differs in C_2 by having a keto group) does not depend on insulin for its transport into the cell.

Insulin also stimulates the uptake of amino acids by the cells. As in the case of glucose, uptake of amino acids is also independent of their subsequent utilization in the cell. Insulin not only facilitates transport of amino acids into cells, but also stimulates their incorporation into protein. The effect is exerted at the ribosomal level.

Insulin is said to stimulate the activity of the enzymes hexokinase and glycogen synthetase. The effects are not demonstrable in vitro on purified enzyme preparations.

It is said to stimulate oxidative phosphorylation in mitochondria of muscle. In the adipose tissue, it antagonizes epinephrine and glucagon action by suppressing fatty acid release by that tissue. The action may be two fold :

(*i*) By increasing glycolysis, more glycerophosphate is made available from glucose. This is used for synthesis of triglyceride from fatty acids.

(*ii*) By reducing the level of cyclic AMP in adipose tissue it inhibits lipolysis and fatty acid release.

Insulin stimulates the entry of Na^+, K^+ and phosphate into adipose tissue cells. This action is independent of glucose utilization by those cells.

2. Liver : The liver cell membrane is freely permeable to glucose and the concentration of glucose within the cells is same as in the extracellular fluid surrounding it. Hence the action of insulin on cell membrane has no relevance to liver cell.

Insulin is said to act on the hepatic cell by increasing the synthesis of the glucokinase, phosphofructokinase, and pyruvate kinase—enzymes concerned in glycolysis—and by repressing the synthesis of pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose-1, -6 diphosphatase and glucose-6-phosphatase enzymes concerned in gluconeogenesis.

The net result of these actions is a decreased glucose output by the liver due to an increased glycolysis and decreased gluconeogenesis. The cyclic AMP level of liver is decreased. Insulin bound to the cell membrane will stimulate the action of membrane bound cyclic AMP-phosphodiesterase

and inhibit the stimulatory effects of the hormones on adneyl cyclase activity (epinephrine and glucagon). The result is a decrease in the cyclic AMP levels in the cell.

The action on hepatic enzymes probably depends on a single factor action on protein kinases resulting in the formation of dephosphorylated enzymes (glucagon and epinephrine cause formation of phosphorylated enzymes).

Insulin is thus an anabolic hormone causing increased carbohydrate metabolism, glycogen formation, lipid synthesis, amino acid uptake and protein synthesis.

Assay of insulin : A unit of insulin is the amount required to reduce the blood glucose level of a normal 24 hour fasted 2 kg. rabbit from 120 mg to 45 mg/100 ml.

15.9 Oral Contraceptives or Pills

It is abundantly clear that the rapidly expanding population of the earth is one of the man's most serious problems. Therefore, it is an ominous prognosis for the future of the human race unless the means of fertility suppressions are made available and used widely throughout the world, especially in the underdeveloped countries like India. Various methods are available for controlling fertility. Out of these, the use of oral contraceptives has been striking successful in controlling fertility.

Oral contraceptives available in the market are mainly of three types :

(a) The most generally used contraceptives contain progestonal agents along with a small amount of estrogenic agents. The latter help to control the duration of the cycle and the menstrual flow.

The contraceptives of this type have at least three points of attack :

(*i*) Those inhibit ovulation by blocking the release of LH,

(*ii*) Those render the cervical mucus hostile to sperm penetration, and

(*iii*) Those induce endometrial changes considered unsuitable for implantation.

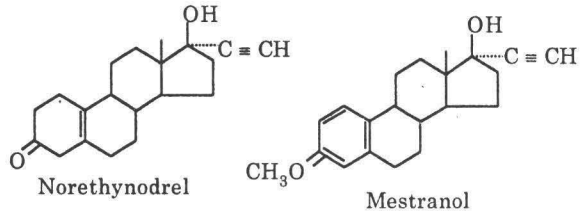
(b) Another type of contraceptives employ the combination of progestrogen and sequential agent. These contraceptives inhibit ovulation by suppressing the release of both FSH and L.H. No other point of interference with the cycle has been determined.

(c) The third type of contraceptives is based upon low-level luteal supplementation. These contraceptives control without inhibiting ovulation. It is more probable that the effect is due to changes induced in the endometrium and/or cervical mucus, rendering them hostile to the sperm penetration. It is also possible that these agents affect the motility of the ovum in the fallopian tube, so that the fertilised ovum reaches the uterine cavity prior to the time in which implantation conditions are optimal.

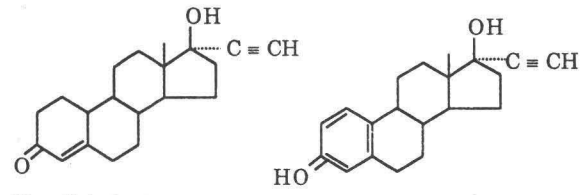
Some of the important oral contraceptive preparations are mentioned below :

Sl.No.	*Trade name*	*Progestrin*	*Oestrogen*
1.	Enovid	Norethynodrel (9.85 mg)	Mestranol (0.15 mg)
2.	Orthonovum	Norethindrone (10mg)	Mestranol (0.00 mg)
3.	Norelestrin	Norethindroneacetate (2.5 mg)	Ethynyl estradiol (0.05 mg)
4.	Ovulen	Ethynodiol diacetate (0.1 mg)	Mestranol (0.1 mg)
5.	Oracon	Dimethisterone (25 mg)	Ethynyl estradiol (0.1 mg)

The structures of some of the components of oral contraceptives are as follows :



Norethynodrel Mestranol



Norethindrone Ethynylestradiol

Prescribed dose : One tablet of the prescribed dose of the combination type contraceptives per day ordinarily is given. The patient begins taking the compound on the 5th day of her menstrual cycle, counting the first day of the menses as day 1. She takes 1 tablet for 20 consecutive days and stops. The drug is often prescribed to be taken with the evening meal.

Side effects : From 15 to 35% of the women using the-oral contraceptives experience undesirable side effects. These signs and symptoms are similar to those usually occurring during pregnancy. Nausea, vomiting, breast fullness, mastalgia, headache, dizziness, depression, apathy, fatigue, pelvic pain and chloasma are the most frequent. Fluid retention and weight gain are also observed. These may be due to a mild increase in aldosterone secretion by the adrenals or to some anabolic activity inherent in the steroid.

16 Vitamins

16.1 History of Discovery

It was Sir Frederick Crowl and Hopkins who reported the necessity of certain accessory food factors in 1880 besides the well-known dietary compounds. However, in 1912, Funk reported for the first time that certain compounds are present in the food which prevent *beriberi, scurvy, pellagra, rickets,* etc. According to him, all these compounds have nitrogen and hence due to their vital function and basic in nature he proposed the name 'vitamin' (L. vita, life + amine). At a later stage it was found that all such compounds are not having nitrogen and therefore the final 'e' was dropped, *i.e.,* the *vitamine* was modified to vitamin.

16.2 Definition of Vitamins

The term "vitamin" *refers to an accessory indispensable food factor, organic in nature (organic acids, amino acids, esters, alcohols, steroids), required by an organism in small amounts to maintain normal growth and regulation of metabolism.*

Generally vitamins are synthesised by plants and are found in animals as a result of food intake or the activity of the micro-organims in the gut. Further, vitamin D may be produced in the skin by irradiation (ultraviolet) of sterols.

The role of vitamins in animal body is not definitely established. Vitamins are not the building units of the animals body. Also, they are not the sources of energy. However, certain vitamins become part of the enzyme systems which are actively involved in enzyme action. Further, all

vitamins have one function in common, *i.e.*, they are growth factors. Lack of any of them from the diet of the young animals slows down or prevents growth. The deficiency of vitamins causes specific diseases like *xerophthalmia, beriberi, scurvy, rickets,* etc. However, these deficiency diseases can be cured or prevented by administration of the vitamin rich diet.

Neither all living organisms require vitamins (some bacteria do not) nor do they necessarily need the same number or kind, since some vitamins can be synthesised by the organism. For example, the guinea pig, man and other primates cannot synthesise vitamin C whereas the rat is able to synthesise it, and therefore does not require it in the diet. Further, it is interesting to note that a particular substance may be a vitamin for one living being but may not be vitamin for another one.

From the point of view of chemical structure, there is very little common to the various vitamins. In general, fresh and natural foods contain all the necessary vitamins in appropriate amounts.

16.3 Classification and Nomenclature of Vitamins

There are about twenty-five vitamins which have been arbitrarily classified into two types :

(*a*) *Fat soluble group :* This type includes vitamin A, D, E and K.

(*b*) *Water-soluble group :* This type includes rest of the vitamins like vitamins of B group, vitamin C, etc. Vitamins are designated by alphabets like A, B, C, D, E, etc. in the order of their discovery : Furthermore, the subgroup of an individual vitamin is designated by the subscripts (*e.g.*, A_1, A_2, B_1, B_2, B_6, B_{12}, D_1, D_2, etc.). However, vitamin H (Biotin) is an exception as it is insoluble both in fat and in water.

16.4 Sources of Vitamins and their Deficiency Diseases

The vitamins so far discovered with their sources and deficiency they cause are given in the Table 16.1.

Table 16.1

Vitamin name	*Source*	*Deficiency disease*
1	*2*	*3*
1. Vitamin A_1	Fish oil, particularly in shark liver oil	Xerophthalmia, *i.e.*, hardening of the cornea
2. Vitamin A_2	Liver of fresh water fish	
3. Vitamin B_1	Rice polishings, liver, kidney, yeast, milk, groundnuts, eggs, green vegetables, and dairy products (except butter)	Beri-beri
4. Vitamin B_2	Yeast, vegetables, milk, egg white, liver, kidney, meat	Dark red tongue, dermatitis and cheilosis
5. Pantothenic acid (B_3)	Liver, kidney, heart spleen, brain, pancreas, tongue, and spleen	Burning sensation, muscle weakness, abdominal disorder and general depression
6. Folic acid (B_4)	Green leafy vegetables and fruits	Macroyclic anaemia (deficiency of R.B.C.)

1	*2*	*3*
7. Nicotinic acid (vitamin B_5)	Plant and animal tissues, meat products like live⁻, meat and kidney, yeast, grain, cereals, pulses, groundnut and coffee	Lesions of those parts of the body which are exposed to sun light
8. Pyridoxin (vitamin B_6)	Ceral grains, molasses and yeast	Severe dermatitis; extreme deficiency causes convulsions
9. Biotin (vitamin H)	Yeast, liver, kidney, and milk	Dermatitis, loss of hair and progressive paralysis
10. Vitamin B_{12}	All animal tissues especially in the liver of ox, sheep, pig, fish, etc.	Pernicious anaemia
11. Vitamin C	Citrus fruits, green vegetables, etc.	Scurvy
12. Vitamin D	Cod liver oil	Rickets
13. Vitamin E	Wheat germ oil, cotton seed oil, soyabean oil and palm oil rice	Antisterility, increase in the number of leucocytes, increases concentration of RNA and DNA in the bone marrow
14. Vitamin K	Cereals; leafy tissues	Hameorrhagic conditions
15. Vitamin P	Grape fruit, orange, lemon etc.	Haemorrhagic conditions

16.5 Provitamins or Precursors

There are some biological inactive compounds which are quite similar to the vitamins and are converted easily into vitamins in vivo. Such compounds are known as provitamins or precursors. For example,

(*i*) β-Carotene is the provitamin for vitamin A, and

(*ii*) Ergosterol is the provitamin for vitamin D_2.

16.6 Biological Functions (Physiological Function) of Vitamins

From the point of view of chemical structure, there is very little common to the various vitamins. But from the point of view of chemical reactions, they have one feature in common, *i.e.*, their ability to take part in reversible oxidation–reduction processes. Thus, vitamins form a part of various coenzymes. The biological activity of vitamins is mainly due to their coenzyme nature, *i.e.*, they act as the cofactors for various enzymes which take part in many biological reactions. Further, it is found that a single vitamin may act as the coenzymes of various enzymes. For example, nicotinamide is present in coenzymes I and II (NAD^+ and $NADP^+$) riboflavin is present in flavine adenine dinucleotide, pantothenic acid is present in co-enzyme A and pyridoxal phosphate is the coenzyme of transaminase. It will be seen that the major deficiency symptoms associated with most of the vitamins are not explained simply by knowledge of the biochemical functions that the related coenzymes perform.

In the Table 16.2, the relationship between vitamins and coenzymes along with the metabolic reaction in which they are participating has been given.

Table 16.2

Vitamin name	*Name of the coenzyme*	*Types of reactions*
1. B_1	Thiamine pyrophosphate	Decarboxylation of α-keto acids
2. B_2	Flavin mono nucleotide and flavin adenine dinucleotide	Oxidation-reduction reactions
3. B_6	Pyridoxal phosphate	Transmination of amino acids and decarboxylation
4. Nicotinamide	DPN, TPN	Oxidation-reduction reactions
5. Pantothenic acid	Coenzyme A	Transference of acetyl groups
6. Biotin	Biotin	Carbon dioxide fixation reaction
7. Folic acid	Tetrahydrofolic acid	Reactions which involve single carbon compounds
8. B_{12}	Cobamide coenzymes	Carbon chain isomerisation

16.7 General Structures of Vitamins

The vitamins have a variety of structures which belong to various classes of organic compounds as shown below :

(1) Vitamin A : It is a diterpenoid.

(2) Vitamin B_1 (Thiamine) : It is a heterocyclic compound having the thiazole nucleus and a pyrimidine nucleus.

(3) Vitamin B_2 (Riboflavin) and Folic acid (a growth factor) : Both have a pteridine nucleus.

(4) Vitamin B_6 : It has a pyridine nucleus.

(5) Vitamin B_{12} : It is the first organic natural product which is known to contain cobalt. It has corrin as a major structural feature.

(6) Vitamins C and D : They are grouped with carbohydrates and steroids respectively.

(7) Vitamin E (Tocopherols) : It has the chromane ring system with an isoprenoid side chain.

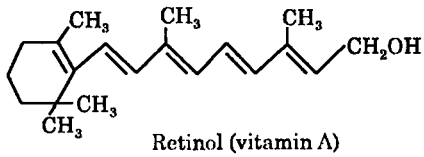
(8) Vitamin K : It is a naphthaquinone derivative.

16.8 Vitamin A or Vitamin A_1 or Retinol or Axerophthol

Introduction : The existence of the fat-soluble factor vitamin A, which occurs in butter, eggs and cod-liver oil was established in 1915 by feeding experiments. It is fat soluble substance. Several naturally occurring compounds having vitamin A skeleton like β-carotene are converted *in vivo* into this vitamin, *i.e.,* vitamin A.

There are two vitamins A_1 and A_2. Usually, when we talk about vitamin A, we mean that it is vitamin A_1. The IUPAC name of vitamin A or A_1 is retinol; that of the corresponding aldehyde is retinal and that of the corresponding acid is retinoic acid.

Only retinol has full vitamin A activity. The term retinoids has been used to describe both the natural forms and synthetic analogs of retinol.



Retinol (vitamin A)

Vitamin A_1 was recognised as early as 1913 by McCollum and Davis.

Occurrence : Vitamin A_1 is a fat soluble vitamin which occurs free and as esters in fats, in fish livers and in blood. The other sources are carrots, green vegetables, sweet potatoes, tomatoes, apricot, salad and cabbage in which it is present in the form of carotenes (provitamins) which are converted into vitamin A_1 in the intestinal tract of animals.

In vegetables, vitamin A exists as a *provitamin* in the form of the yellow pigment β-*carotene*, which consists of two molecules of retinal joined at the aldehyde end of their carbon chains. However, because β-carotene is not efficiently metabolized to vitamin A, weight for weight, β-carotene is only one-sixth as effective a source of vitamin A as retinol.

Physiological functions of vitamin A_1 in vision : The retinas of most animals have photosensitive pigment such as rhodopsin. The light-absorbing group of this pigment is the polysaturated aldehyde 11-*cis*-retinal.

When light falls on the retina of the eye, the chemical changes take place in retina in which absorption of light takes place by the conjugated polyene system of 11-*cis*-retinal and the interconversion of *cis-trans* isomers. The visual process gets started as soon as rhodopsin absorbs a photon of light and the following two phenomena may take place.

(*i*) First of all, 11-*cis*-retinal of rhodopsin gets isomerised to the all-trans-form.

(*ii*) Then as the all-*trans* form of retinal does not fit precisely the site on the surface of the protein, there occurs the hydrolysis of the —Ch=N— group to afford opsin and the all-retinal. The absorption maximum of rhodopsin is 500 nm, giving it its bright red color which gets lost on its light-initiated transformation to all-*trans*-retinal (vitamin A aldehyde) and opsin which together have an absorption maximum at 387 nm and are thus yellow. Further bleaching to a colourless form occurs when the all-*trans*-retinal (vitamin A aldehyde) gets reduced enzymatically to all *trans-vitamin* A.

Enzymes present in the liver get transformed all-*trans*-vitamin A into 11-*cis* vitamin A and the latter is then returned to the eye where it gets reoxidised to 11-*cis*-retinal which is used for the synthesis of rhodopsin (Fig. 16.1).

Retinol and retinal are interconverted in the presence of NAD or NADP- requiring dehydrogena ses or reductases, present in many tissues. However, *once formed from retinal, retinoic acid cannot be converted back to retinal, or to retinol.* Thus, retinoic acid can support growth and differentiation *but cannot replace retinal in its role in vision or retinol in its support of the reproductive system.*

As a steroid hormone : When retinol is taken up into CRBP it is transported about the cell and binds to *nuclear proteins,* where it is probably involved in the *control of the expression of certain genes.* Thus, in this respect vitamin A behaves in a manner similar to that of steroid hormones. The requirement of vitamin A for normal reproduction may be ascribed to this function.

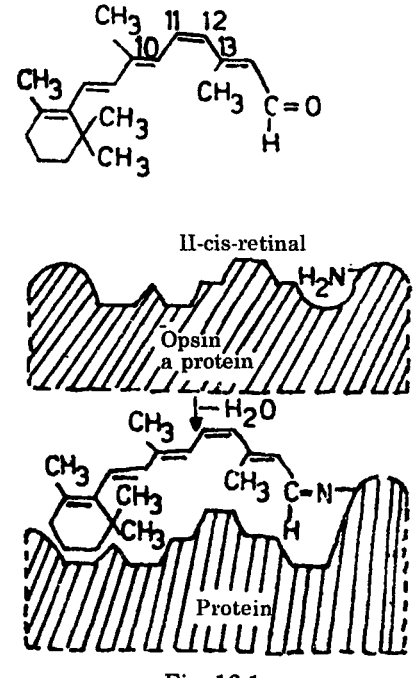


Fig. 16.1.

Digestion of vitamin A_1 : Retinol esters dissolved in the fat of the diet are dispersed in bile droplets and hydrolyzed in the intestinal lumen, followed by absorption directly into the intestinal epithelium. Ingested *β-carotenes* may be oxidatively cleaved by *β-carotenedioxygenase* (Fig. 16.2).

This cleavage utilizes molecular oxygen and is enhanced by the presence of bile salts, and generates 2 molecules of *retinaldehyde (retinal).* Also, in the intestinal mucosa, retinal is reduced to retinol by a specific *retinaldehyde reductase* utilizing NADPH. A small fraction of the retinal is oxidized to *retinoic acid.* Most of the retinol is esterified with saturated fatty acids and incorporated into lymph chylomicrons, which enter the bloodstream. These are converted to chylomicron remnants, which are taken up by the liver together with their constant of retinol. Carotenoids may escape some of these processes and pass directly into the chylomicrons.

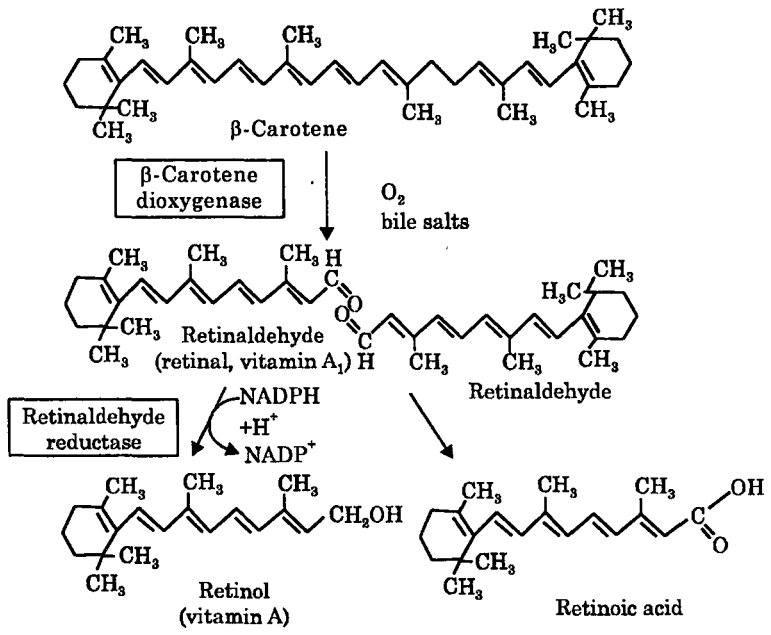


Fig. 16.2. β-Carotene and its cleavage to retinaldehyde. The reduction of retinaldehyde to retinol and the oxidation of retinaldehyde to retinoic acid are also shown.

Storing of vitamin A_1 : In the liver, vitamin A is stored as an ester in the lipocytes, probably as a lipoglycoprotein complex. For transport to the tissues, it is *hydrolyzed* and the retinol bound to apo-tetinol binding protein (RBP). The resulting *holo-RBP* is processed in the Golgi apparatus and secreted into the plasma. Retinoic acid is transported in plasma bound to albumin. Once inside extrahepatic cells, retinol is bound by *a cellular retinol binding protein* (CRBP).

Vitamin A toxicity occurs after the capacity of RBP has been exceeded and the cells are exposed to *unbound retinol.*

Properties : When the vitamin A_1 was isolated originally, it was a yellow oil. But later on it was isolated as a crystalline solid, m.p. 63-64°C. It is optically inactive and sensitive to light and air but is resistant to heat. It is destroyed by UV light.

Daily requirements : Biological activity of pure vitamin is 3300 I.U. per mg. Human system requires about 5000 I.U. per day for normal growth. During pregnancy it is increased to about 6000 to 8000 I.U. For infant it is about 1500 I.U. per day.

Deficiency diseases : The deficiency of this disease causes several abnormalities in the body. Some important of them are given below.

Night blindness : Lack of vitamin A causes characteristic deficiency symptoms. These are due to malfunction of the various cellular mechanisms in which retinoids participate. One of the first indications of vitamin A deficiency is *defective night vision,* which occurs when liver stores are nearly exhausted. Further depletion leads to *keratinization* of epithelial tissues of the eye, lungs, gastrointestinal, and genitourinary tracts, coupled with reduction in mucous secretion. Deterioration in the tissues of the eye, *xerophthalmia,* leads to blindness. Vitamin A deficiency occurs mainly on poor basic diets coupled with a lack of vegetables that would otherwise provide the provitamin, β-carotene.

(i) Xerophthalamia : Its deficiency in animals may cause night blindness but a prolonged deficiency of this may lead to hardening of the conjunctive, softening of cornea which is termed as xerophthalmia and may lead to complete blindness in some cases.

(ii) Dermatitis : The skin becomes dry, resulting scaliness condition and looks like toad skin. The hair also become dry.

(iii) Skeleton : Its deficiency causes the abnormal growth of the bones such as skull, vertebral column.

(iv) Salivary and mucous glands : The ability of salivary glands to secrete saliva is lost. Similarly, mucous gland cells of the guts are filled with bacteria.

(v) Resistive nature : Vitamin A_1 is responsible to make the body resistance to infection. Its deficiency reduces this ability of the body.

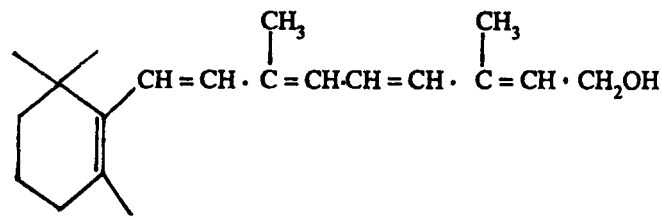
Excess : While an adequate supply of vitamin A_1 is required for the proper health of animals, excess of this vitamin can be injurious. Some symptoms of vitamin A_1 excess are bone fragility, nausea, weakness, liver enlargement, pains in bones and joints, loss of hair and dermatitis.

16.9 Vitamin A_2 (3, 4-Dehydroretinol)

The vitamin is similar to vitamin A_1 but possesses one more conjugated double bond in its structure than that of vitamin A_1.

Vitamin A_2 is also known as retinol$_2$. It is found in certain fish oils, especially fresh water fish.

Vitamin A_2, *m.p.* 63—65°C, is the *all-trans* isomer.



Vitamin A_2

Its importance in the animals is not known.

16.10 Vitamin B Complex

It is not one vitamin but a group of water soluble vitamins which are found in yeast, liver, rice polishings, etc. This group of vitamins includes (*i*) thiamine (B_1), (*ii*) riboflavinn (B_2), (*iii*) pantothenic acid, (*iv*) nicotinic acid (B_5), (*v*) pyridoxine (B_6), (*vi*) folic acid (Bc), (*vii*) biotin, (*viii*) cyanocobalamine (B_{12}). Other compounds which have definitely been isolated from the vitamin B complex are (*i*) *p*-aminobenzoic acid (a growth factor for bacteria), (*ii*) myoinositol (a growth factor in animals), (*iii*) chlorine, (*iv*) carnitine (oxidation of falty acids in certain insects and lipoic acid (a growth factor for some micro organisms).

Because of their water solubility, excesses of these vitamins are excreted in urine and so rarely accumulate in toxic concentrations. For the same reason, their storage is limited (apart from cobalamin) and as a consequence they must be provided regularly.

16.11 Vitamin B_1 or Thiamin or Aneurin

Occurrence : It is a water soluble vitamin which occurs abundantly in the outer coats of the seeds of many plants including the cereal grains like rice, wheat, etc. In small quantity, it is also found in some animal organs, *viz.*, liver and kidney. It also occurs in yeast, milk, ground nuts, eggs, all green vegetables, root, fruits, and dairy products (except butter). In animal tissues and in yeast it occurs primarily as the coenzyme thiamine pyrophosphate or cocarboxylase.

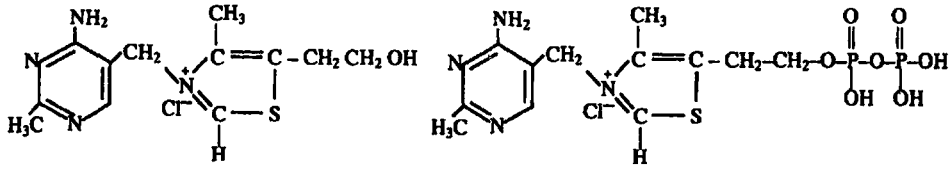


Fig. 16.3. In thiamin diphosphate, the OH group is replaced by pyrophosphate.

Structure : Thiamin consists of a substituted pyrimidine joined by a methylane bridge to a substituted thiazole. Active thiamin is thiamin diphosphate. An ATP-dependent thiamin diphosphotransferase present in brain and liver is responsible for the conversion of thiamin to its active form, thiamin diphosphate (pyrophosphate) (Fig. 16.3).

Functions : Thiamin diphosphate serves as a coenzyme in enzymatic reactions, transferring an activated aldehyde unit. There are two types of such reactions : (1) an *oxidative decarboxylation* of α-keto acids (*e.g.*, α-ketoglutarate, pyruvate, and the α-keto analogs of leucine, isoleucine, and valine); and (2) *transketolase reactions* (*e.g.*, in the pentose phosphate pathway). All of these reactions are *inhibited in thiamin deficiency*. In each case, the thiamin diphosphate provides a reactive carbon on the thiazole that forms a carbanion, which is then free to add to the carbonyl group of, for instance, pyruvate. The addition compound then decarboxylates, eliminating CO_2. This reaction occurs in a multienzyme complex known as the pyruvate dehydrogenase complex.

The oxidative decarboxylation of α-ketoglutarate to succinyl-CoA and CO_2 is catalysed by an enzyme complex structurally very similar to the pyruvate dehydrogenase complex. Again, the thiamin diphosphate provides a stable carbanion to react with the α-carbon of α-ketocarboxylic acid derivaties of the branched-chain amino acids utilizes thiamin diphosphate. The role of thiamin diphosphate as a coenzyme in the transketolase reactions is very similar to that described above for the oxidative decaroxylations.

Deficiency diseases : A deficiency of this vitamin in man produces the classic disease known as beri-beri (a type of paralysis). Beri-beri is of two types :

(a) Dry beri-beri : This takes place in the case of lesser deficiency of thiamine. In this type of disease, there occurs muscular weakness and loss of weight, neuritis, pain in the arms and legs and decrease in blood pressure. The person suffering from dry beri-beri responds rapidly to thiamine administration.

(b) Wet beri-beri : This takes place in the case of severe deficiency of thiamine. In this case, the entire nervous system is affected and results in a type of paralysis, leading to edema and impaired cardiac function. The wet type of beri-beri is more common in infants.

16.12 Vitamin B_2 or Riboflavin or Lactoflavin

Introduction : Since vitamin B_2 is chemically related to the yellow water soluble pigments known as *flavins* and it was also isolated from milk, it is also known as *lactoflavin*.

Riboflavin consists of a heterocyclic isoalloxazine ring attached to the sugar alcohol, ribitol. It is a coloured, fluorescent pigment that is relatively heat stable but decomposes in the presence of visible light. *Active Riboflavin is Flavin Mononucleotide (FMN) and Flavin Adenine Dinucleotide (FAD).*

FMN is formed by ATP-dependent phosphorylation of riboflavin (Fig. 16.4), whereas FAD is synthesized by a further reaction with ATP in which the AMP moiety of ATP is transferred to FMN (Fig. 16.5).

Occurrence : Vitamin B_2 is widely distributed in plants and animals. It is present in yeast, vegetables, milk, egg white, liver kidney, meat, etc. The primary source of vitamin B_2 is plant material, although commercial production by yeasts and certain micro organisms is practised.

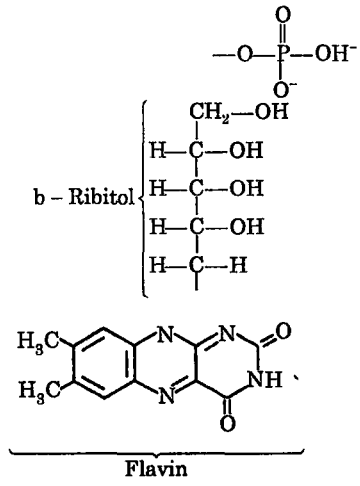


Fig. 16.4. Riboflavin. In riboflavin phosphate (flavin mononucleotide, FMN), the –OH is replaced by phosphate.

The vitamin B_2 occurs in nature almost exclusively as a constituent of the two flavin prosthetic groups, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD).

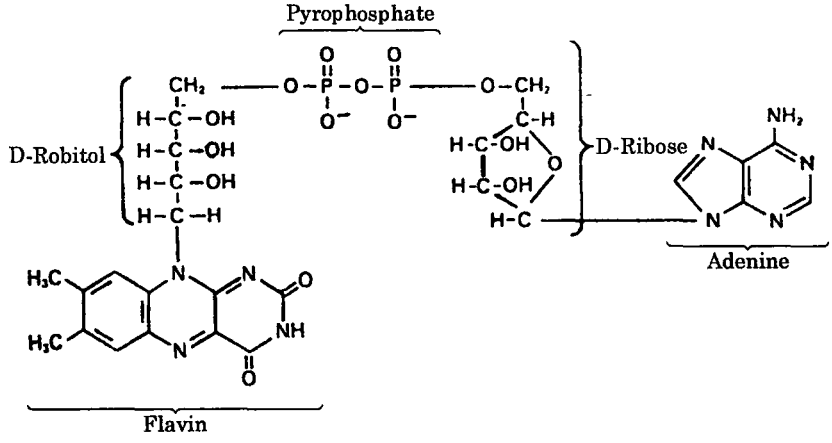


Fig. 16.5. Flavin adenine dinucleotide (FAD).

FMN and FAD serve as prosthetic groups of oxidoreductase enzymes. These enzymes are known as *flavoproteins*. The prosthetic groups are usually tight but not covalently bound to their apoproteins. Many flavo protein enzymes contain one or more metals, *e.g.*, molybdenum and iron, as essential cofactors and are known as *metalloflavoproteins*.

Flavoprotein enzymes are widespread and are represented by several important oxidoreductases in mamalian metabolism, *e.g., α-amino acid oxidase* in amino acid deamination, *xanthine oxidase* in purine degradation, *aldehyde dehydrogenase* in the degradation of aldyhydes, *mitochondrial glycerol-3-phosphate dehydrogenase* in transporting reducing equivalents from the cytosol into mitochondria, *succinate dehydrogenase* in the citric acid cycle, *acyl-CoA dehydrogenase* and the *electron-transferring flavoprotein* in fatty acid oxidation, and *dihydrolipoyl dehydrogenase* in

the oxidative decarboxylation of pyruvate and α-ketoglutarate; *NADH dehydrogenase* is a major component of the respiratory chain in mitochondria. All of these enzyme systems are impaired in riboflavin deficiency.

In their as coenzymes, flavoproteins undergo reversible reduction of the isoalloxazine ring to yield the reduced forms $FMNH_2$ and $FADH_2$.

Deficiency disease : In view of its widespread metabolic functions it is surprising that riboflavin deficiency does not lead to major life-threatening conditions. However, when there is deficiency, various symptoms are seen, including angular stomatitis, cheilosis, glossitis, seborrhea, and photophobia.

Riboflavin is synthesized by plants and microorganisms, but not by mammals. Yeast, liver, and kidney are good sources of the vitamin, which is absorbed in the intestine by a phosphorylation—de-phosphorylation sequence in the mucosa. Hormones (*e.g.*, thyroid hormone and ACTH), drugs (*e.g.*, chlorpromazine, a competitive inhibitor), and nutritional factors affect the conversion of riboflavin to its cofactor forms. Because of its light sensitivity, riboflavin deficiency may occur in newborn infants with hyperbilirubinemia who are treated by phototherapy.

The average healthy adult human requires 2-3 mg of vitamin B_2 per day.

Properties : Vitamin B_2 is a bright yellow powder which decomposes at ~280°C. It is soluble in water and in ethanol but is insoluble in chloroform and other organic solvents.

As the aqueous solution of vitamin B_2 shows a yellowish-green flourescence having λ_{max} 565 nm, this property is employed as a means of estimating vitamin B_2.

16.13 Vitamin B_3 or Pantothenic Acid

Occurrence : As vitamin B_3 is almost of universal occurrence, it is named as pantothenic acid (Pantothenic, a Greek word, which means from "everywhere"). It occurs in all types of animal tissues as a component of coenzyme A and of acyl-carrier protein (ACP). The richest sources of vitamin B_3 are liver, kidney, heart, brain, pancreas, tongue and spleen. Vitamin B_3 is also produced by some moulds and green plants.

Pantothenic acid is formed by combination of pantoic acid and β alanine (Fig. 16.6).

Active Pantothenic Acid is Coenzyme A (CoA) and the Acyl Carrier Protein (ACP)

Patothenic acid is absorbed readily in the intestines and subsequently phosphorylated by ATP to form 4′-phosphopantothenate. Addition of cystein and removal of its carboxyl group results in the net addition of thioethanolamine, generating *4′-phosphopantetheine,* the prosthetic group of both CoA and ACP. Like the active coenzymes of so many other water-soluble vitamins, CoA contains an adenine nucleotide. Thus, 4′-phosphopantetheine is adenylylated by ATP to form *dephospho-CoA.* The final phosphorylation occurs with ATP adding phosphate to the 3′-hydroxyl group of the ribose moiety to generate CoA (Fig. 16.6).

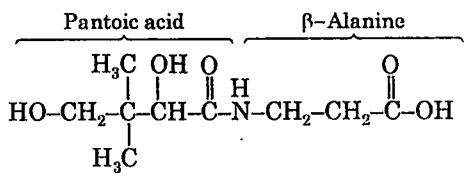


Fig. 16.6. Pantothenic acid.

Thiol group acts as a carrier of acyl radicals in both CoA and ACP.

$HOCH_2C(CH_3)_2CHOH\ CONHCH_2CH_2COOH$

Pantothenic acid

Deficiency disease : The symptoms of vitamin B_3 deficiency in man are not known with certainty. However, its deficiency in many may cause burning sensation, muscle weakness, abdominal disorder and general depression.

Vitamin B_3 is very essential for chicks, rats and fowls. Its deficiency in rats causes retardation of growth, depigmentation of the fur and spectaled eye condition. Vitamin B_3 is also capable of promoting the growth of yeast and of bacteria.

Properties : Vitamin B_3 is a pale yellow oil which is sensitive to heat, to acids and to bases. This is optically active. The compound mainly shows acidic properties.

16.14 Folic Acid or Vitamin B_4

Occurrence : Folic acid and its derivatives, which are chiefly the tri and hepta glutamyl peptides, are widespread in nature. The vitamin occurs in green leafy vegetables and fruits and hence its name (Latin-folium = leaf). In animals, it is present in liver and kidney. It also occurs in yeast. Among vegetables and fruits, it mainly occurs in lemons, bananas, spinach, asparagus and strawberries.

In animals liver, folic acid is pteroglutamic acid whereas in yeasts and other sources it exists in forms which contain three or seven glutamic acid residues.

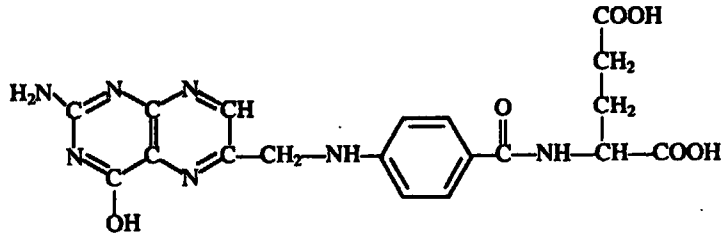


Fig. 16.7. The structure and numbering of atoms of folic acid.

Folic acid, or folate, consists of the base *pteridine* attached to one molecule each of *p-aminobenzoic acid* (PABA) and *glutamic acid* (Fig. 16.7). Animals are not capable of synthesizing PABA or of attaching glutamate to pteroic acid and, therefore, require folate in their diet; yeast, liver, and leafy vegetables are major sources. In plants, folic acid exists as a polygutamate conjugate consisting of a γ-linked polypetide chain of 7 glutamate residues. In the live, the major folate is a pentaglutamyl conjugate.

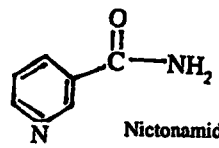
Active folate is tetrahydroloate (H_4 folate). Folate derivatives in the diet are cleaved by specific intestinal enzymes to monoglutamyl folate for absorption. Most of this is reduced to *tetrahydrofolate* in the intestinal cell (Fig. 16.8) by the enzyme *folate reductase,* which uses NADPH as donor of reducing equivalent. Tetrahydrofolate polyglutamates are probably the functional coenzymes in tissues.

Deficiency disease : Folic acid is essential for a growth of a number of micro-organisms and is effective in the treatment of certain types of anaemia in which the size of red blood corpuscles is larger than the normal one. It may be accompanied by diarrhoea, gastrointestinal disorders and glossitis.

Properties : It is yellow crystalline solid which is sparingly soluble in water. This is stable in acid solution but is sensitive to sunlight and to high temperature.

16.15 Nicotinic Acid (Niacin) or Vitamin B_5 and Nicotinamide (Niacin Amide)

Introduction : The term niacin is the official name of the vitamin nicotinic acid. The biochemically active form of the vitamin niacin is the amide, *nicotinamide or niacinamide.* Nicotinic acid is a monocarboxylic acid derivative of pyridine.



Nictonamide

Nicotinate is the form of niacin required for the synthesis of NAD^+ and $NADP^+$ by enzymes present in the cytosol of most cells. Therefore, any dietary nicotinamide must first undergo

deamidation to nicotinate. In the cytosol, nicotinate is converted to *desamido-NAD*$^+$ by reaction first with 5-phosphoribosyl 1-pyrophosphate (PRPP) and then by adenylation with ATP. The amido group of glutamine then contributes to form the coenzyme NAD^+. This may be phosphorylated further to form $NADP^+$.

NAD$^+$ and NADP$^+$ Are Coenzymes of Many Oxidoreductase Enzymes

The nicotinamide nucleotides play a widespread role as coenzymes to many dehydrogenase enzymes occurring both in the cytosol, *e.g., lactate dehydrogenase,* and within the mitochondria, *e.g., malate dehydrogenase*. They are therefore key components of many metabolic pathways affecting carbohydrate, lipid, and amino acid metabolism. Generally, *NAD-linked dehydrogenases* catalyze oxidoreduction reactions in *oxidative pathways, e.g.,* the citric acid cycle, whereas *NADP-linked dehydrogenases* or reductases are often found in pathways concerned with *reductive syntheses, e.g.,* the pentose phosphate pathway.

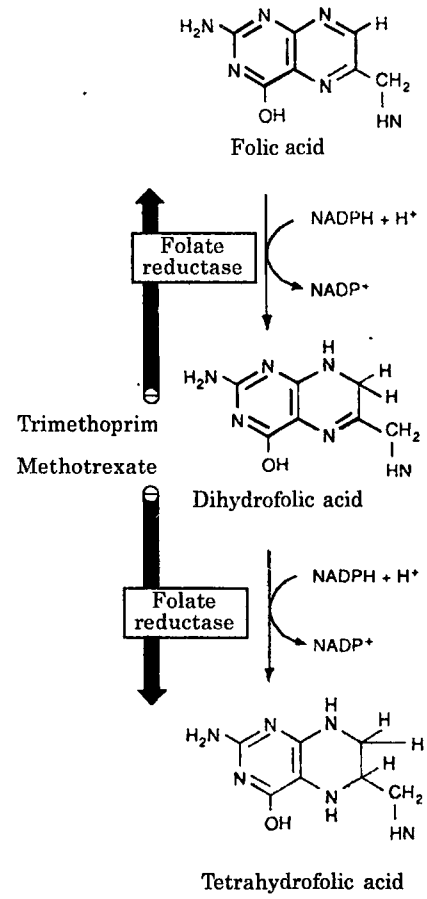


Fig. 16.8 The reduction of folic acid to dihydrofolic acid and dihydrofolic acid to tetrahydrofolic acid by the enzyme folate reductase. Trimethoprim is a selective inhibitor of folate reductase in gram-negative bacteria and has little effect on the mammalian enzyme. It is therefore used as an antibiotic. Methotrexate (amethopterin) binds more strongly and is used as an anticancer drug.

Occurrence : Niacin is widely distributed in plant and animal tissues, meat products like liver, meat and kidney. It is also present in yeast, grain cereals, pulses, groundnut and coffee.

The coenzyme forms of the nicotinamide are the nicotinamide nucleotide coenzymes I and II which play a part in many biological oxidations.

Deficiency disease : Both nicotonic acid and nicotinamide are human pellagara-preventing (P.P) factor. Pellagara is characterised by the lesions of those parts of the body which are exposed to sunlight. Their deficiency also causes black tongue in dogs.

Severe deficiency of both nicotinic acid and nicotinamide causes disturbances in digestive and nervous systems.

Nicotinic acid (but not nictotinamide) has been used therapeutically for lowering plasma cholesterol. This is due to inhibition of the flux of FFA from adipose tissue, which leads to less formation of the cholesterol bearing lipoproteins, VLDL, IDL and LDL.

16.16 Pyridoxine or Adermin or Vitamin B_6

Introduction : Actually vitamin B_6 is not one vitamin but a group of three vitamins, namely, pyridoxine, pyridoxal and pyridoxamine. These three vitamins are interconvertible in the form of their phosphates. All three have equal vitamin activity.

Active vitamin B_6 is pyridoxal phosphate. All forms of vitamin B_6 are absorbed from the intestine, but some hydrolysis of the phosphate esters occurs during digestion. Pyridoxal phosphate is the major form transported in plasma. Most tissues contain the enzyme *pyridoxal kinase,* which is able to catalyze the phosphorylation by ATP of the unphosphorylated forms of the vitamin to their respective phosphate esters (Fig. 16.10). While pyridoxal phosphate is the major coenzyme expressing B_6 activity, pyridoxamine phosphate may also act as an active coenzyme.

Generally, pyridoxine is referred to as vitamin B_6. As this vitamin is antidermatitic factor for rats, it is also known as *adermin.*

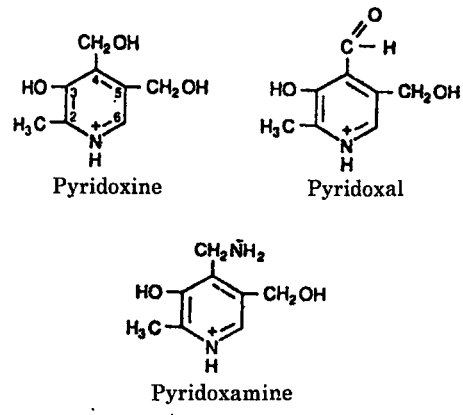


Fig. 16.9. Naturally occurring forms of vitamin B_6.

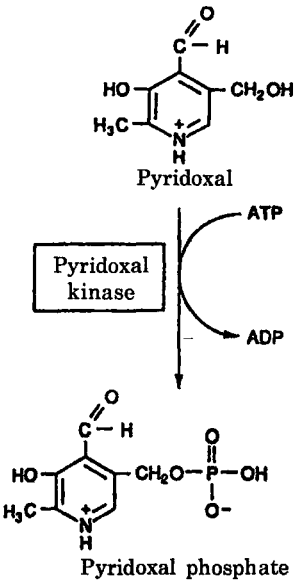


Fig. 16.10. The phosphorylation of pyridoxal by pyridoxal kinase to form pyridoxal phosphate.

Occurrence : The three forms of vitamin B_6 occur abundantly in various plants and animal sources; cereal grains, molasses and yeast are especially rich sources of all the three forms of vitamin B_6. Pyridoxamine also occurs in nature as their phosphate derivatives which are the coenzyme forms of vitamin.

Functions : *1. Pyridoxal phosphate as a coenzyme of several enzymes of amino acid metabolism :* By entering into a *Schiff base* combination between its aldehyde group and the amino group of an α-amino acid (Fig. 16.11), Pyriodoxal phosphate can facilitate changes in the 3 remaining bonds of the α-amino carbon to allow either transamination, decarboxylation, or aldolase activity, respectively.

2. Involvement of pyridoxal phosphate in glycogenolysis : The coenzyme is an integral part of the mechanism of action of phosphorylase, the enzyme mediating the breakdown of glycogen. In this action it also forms an initial Schiff base with an ε-amino group of a lysine residue of the enzyme, which, however, remains intact throughout the phosphorolysis of the 1 ⟶ 4 glycosidic bonds to form glucose 1-phosphate. Muscle phosphorylase may account for as much as 70-80% of total body vitamin B_6.

Deficiency disease : In rats and many other animals, the initial vitamin B_6 deficiency causes severe dermatitits. However, extreme deficiency in these animals causes convulsions similar to those of epilepsy and indicates a profound disturbance in central nervous system. The vitamin B_6 also serves as growth factor for many bacteria.

In man, deficiency of vitamin B_6 causes nervousness, insomnia, irritability, stomatitis (inflammation of the mucous membrane of the mouth) and also causes general weakness. Vitamin B_6 is also employed to stop nausea and vomiting in pregnancy and for treatment of epilepsy and acne or dermatitis.

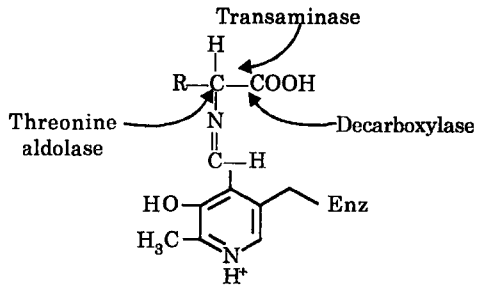
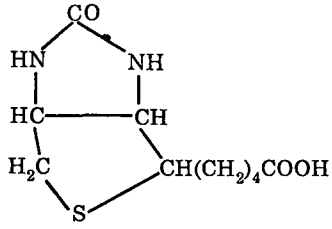


Fig. 16.11. The covalent bonds of an α-amino acid that can be made reactive by its binding to various pyridoxal phosphate specific enzymes.

16.17 Biotins or (Vitamin H)

Introduction : In 1901, Wildiers confirmed that bios, an extract of yeast, was necessary for the growth of yeast. Later, Fulmar *et al.* (1922) reported that two substances were present in bios. In 1923, Miller repeated the earlier experiments and showed that three substances were actually present in bios. The first of these was called *Bios I* which was proved to be *myoinositol,* the second was called *Bios IIA* which proved to be β-*alanine* (Miller, 1936) or *pantothenic acid* (Rainbow *et al.*, 1939) and the third was called *Bios IIB* which was shown to be identical with *biotin,* a substance which was isolated by KÖ gl *et al.* (1936) as the methyl ester from egg yolk.

In 1940, du Vigneaud *et al.* were successful in isolating a substance from liver which possessed the same biological properties as biotin. Later, KÖ gl *et al.* (1943) called biotin obtained from egg yolk as α-*biotin* whereas that obtained from liver as β-*biotin.* Both the compounds possessed the same molecular formula, $C_{10}H_{16}N_2O_3S$. Later Krueger *et al.*, and many other workers repeated that both α-and β-biotins are most probably identical and, therefore, the current practice is to employ the term biotin for β-biotin.



Biotin

Biotin is an imidazole derivative widely distributed in natural foods. As a large protein of the human requirement for biotin is met by *synthesis from intestinal bacteria,* biotin deficiency is caused not by simple dietary deficiency but by defects in utilization.

Functions of Biotin

As a coenzyme of carboxylase coenzymes : Biotin functions as a component of specific multi-subunit enzymes (Table 16.2) that catalyze carboxylase reactions. A carboxylate ion is attached to the N^1 of the biotin, generating an activated intermediate, *carboxybiotin-enzyme.* This step requires HCO_3^-, ATP, Mg^+, and acetyl-CoA (as an allosteric effector). The activated carboxyl group is then transferred to the substrate of the reaction, *e.g.,* Pyruvate.

Table 16.2
BIOTIN-DEPENDENT ENZYMES IN ANIMALS

Enzyme	*Role*
Pyruvate carboxylase	First reaction in pathway that converts 3-carbon precursor to glucose (gluconeogenesis)
	Replenishes oxaloacetate for citric acid cycle
Acetyl-CoA carboxylase	Commits acetate units to fatty acid synthesis by forming malonyl-CoA
Propionyl-CoA carboxylase	Converts propionate to succinate, which can then enter citric acid cycle
β-Methylcrotonyl-CoA Carboxylase	Catabolism of leucine and certain isoprenoid compounds

Occurrence : The three forms of vitamin B_6 occur abundantly in various plants and animal sources; cereal grains, molasses and yeast are especially rich sources of all the three forms of vitamin B_6. Pyridoxal and pyridoxamine also occur in nature as their phosphate derivatives which are the coenzyme forms of vitamin.

Deficiency disease : It rats and many other animals, the initial vitamin B_6 deficiency causes severe dermatitis.

Consumption of Raw Eggs can Cause Biotin Deficiency

Egg white contains a heat-labile protein, *avidin,* which combines very tightly with biotin, preventing its absorption and inducing biotin deficiency. The symptoms include depression, hallucination, muscle pain, and dermatitis. Absence of the enzyme *holocarboxylase synthase,* which attaches biotin to the lysine residue of the carboxylase apoenzymes, also causes biotin deficiency symptoms, including accumulation of substrates of the biotin-dependent enzymes, which can be detected in urine. These metabolites include lactate, β-methylcrotonate, β-hydroxypropionate. In some cases children with this deficiency have immune deficiency diseases.

Properties : β-Biotin exists in fine long needles having decomposition temperature at 230°C. It is optically active and is dextro-rotatory. It is soluble in dilute alkali solution but sparingly soluble in dilute mineral acids.

16.18 Vitamin B_{12} or Cyanocobalamin

Introduction : Vitamin B_{12} is the first natural product which contains cobalt.

Vitamin B_{12} (cobalamin) has a complex ring structure (corrin ring), similar to a porphyrinring, to which is added a *cobalt ion* at its center (Fig. 16.12). The vitamin is synthesized exclusively by microorganisms. Thus, it is absent from plants—unless they are contaminated by microorganisms—but is conserved in animals in the liver, where it is found as methylcobalamin, adenosylcobalamin, and hydroxocobalamin. Liver is therefore a good source of the vitamin, as is yeast. The commercial preparation is cyanocobalamin.

Intrinsic Factor is Necessary for Absorption of Vitamin B_{12}

The intestinal absorption of vitamin B_{12} is mediated by receptor sites in the ileum that require it to be bound by a highly specific glycoprotein, *intrinsic factor,* secreted by parietal cells of the gastric mucosa. After absorption, the vitamin is bound by a plasma protein, *transcobalamin II,* for transport to the tissues. It is stored in the liver, which is unique for a water-soluble vitamin, bound to transcobalamin I.

Active B_{12} Coenzymes are Methylcobalamin and Deoxyadenosylcobalamin

After transport in the blood, free cobalamin is released into the cytosol of cells as

hydroxocobalamin. It is either converted in the cytosol to *methylcobalamin* or enters mitochondria for conversion to 5'-*deoxyadenosylcobalamin.*

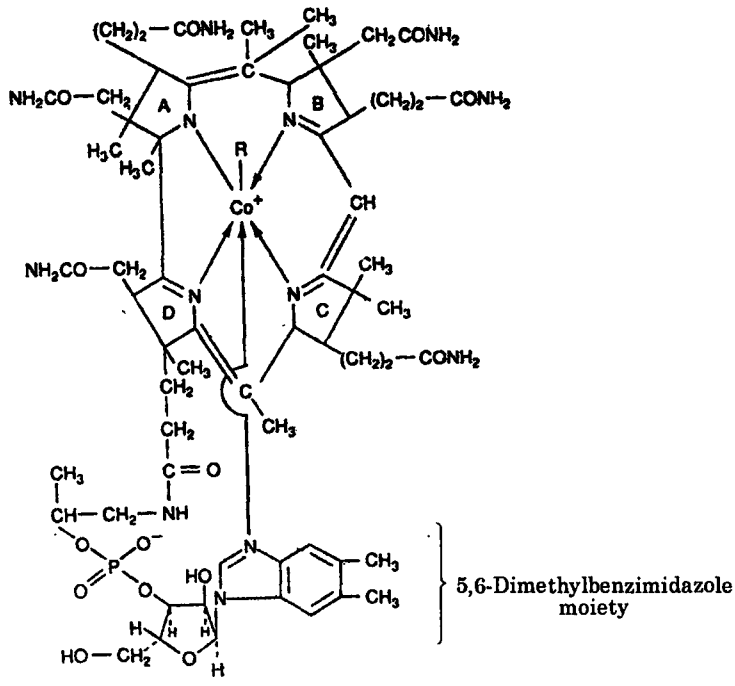


Fig. 16.12. Vitamin B_{12} (cobalamin). R may be varied to give the various forms of the vitamin, *e.g.*, R = CN in cyanocobalamin; R = OH in hydroxocobalamin; R = 5'-deoxyadenosyl in 5'-deoxyadenosylcobalamin; and R = CH_3 in methylcobalamin.

Functions

1. *Deoxyadenosylcobalamin is the Coenzyme for the Conversion of methylmalonyl-CoA to Succinyl-CoA* (Fig. 16.13).

This is a key reaction in the pathway of conversion of propionate to a member of the citric acid cycle and is, therefore, of significance in the process of *gluconegeneis.* It is of particular importance in ruminants, as propionate is a major product of microbial fermentation in the rumen.

2. *Methylcobalamin is Coenzyme in the Combined Coversion of (1) Homocysteine to Methionine and (2) Methyl Tetrahydrofolate to Tetrahydrofolate* (See Fig. 16.13).

In this reaction, the methyl group bound to cobalamin is transferred to homocysteine to form methionine and the cobalamin then removes the methyl group from N^5-*methyltetrahydrofolate* to form tetrahydrofolate. The metabolic benefits of this reaction are that stores of methionine are maintained and tetrahydrofolate is made available to participate in urine, pyrimidine, and nucleic acid syntheses.

Occurrence : Vitamin B_{12} is not present in plant kingdom but is found in all animal tissues especially in the liver of ox, sheep, horse, pig, fish, etc. It is also present in cow dung and urine. It is also synthesised by certain micro-organisms.

Milk, eggs, cheese, and meat contain 1—5 μg/100 g of material.

Deficiency disease : It is essential growth factor for many micro-organisms. Its deficiency in man causes pernicious anaemia which is followed by degradation of spinal cord. This disease does not arise due to the absence of vitamin B_{12} in diet but due to the lack of secretion in stomach

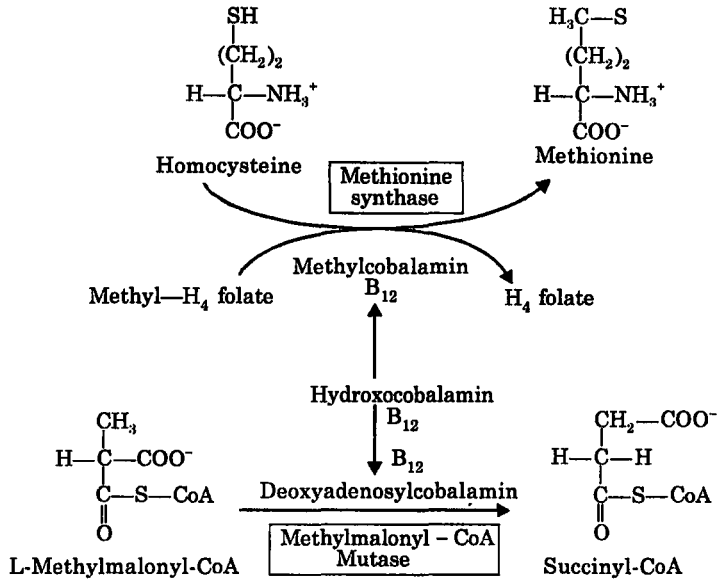


Fig. 16.13. The important reactions catalyzed by vitamin B_{12} coenzyme–dependent enzymes.

called intrinsic factor which is essential for the assimilation of vitamin B_{12}. The same symptoms also appear in the case of folic acid deficiency. Thus, patients respond to either of the two vitamins. However, B_{12} is considered to be more beneficial than folic acid.

Deficiency of Vitamin B_{12} Leads to Megaloblastic Anemia

When absorption is prevented by lack of *intrinsic factor* (or by gastrectomy the condition is called *pernicious anemia.* Vegans are at risk of actual diet deficiency as the vitamin is found only in foods of animal origin or form microorganisms, food contaminated with microorganisms being of benefit from this point of view. The deficiency leads to impairment of the methionine synthesis reaction. Anemia results from impaired DNA synthesis affecting formation of the nucleus of new erythrocytes. This is due to impaired purine and pyrimidine synthesis resulting from tetrahydrofolate deficiency as a consequence of folate being trapped as methyltetrahydrofolate (the "folate trap") (see Fig. 16.13). Homocystinuria and methylmalonic aciduria also occur. The neurologic disorder associated with vitamin B_{12} deficiency may be secondary to a relative deficiency of methionine.

Four inherited disorders of cobalamin metabolism have been described. Two affect synthesis of deoxyadenosylcobalamin only; in the other two, patients are unable to synthesize either deoxyadenosylcobalamin or methylcobalamin.

Properties : Vitamin B_{12} is a red crystalline substance which is slightly soluble in water. It is stable to heat.

Mechanism of action of vitamin B_{12} : The mechanism of action of cobalamin is not known with certainty although it influences several metabolic processes. It affects the synthesis of nucleic acids and metabolism of glycine, serine, methionine and choline. It also works as a coenzyme (commonly called coenzyme B_{12}) in enzymes transferring methyl groups. Its role in transmethylation reaction involving methylated tetrahydro-folic acid has been summarised in Fig. 16.14.

It works as a coenzyme in several other reactions also, like reduction of formate to methyl groups, anaerobic degradation of lysine, reduction of disulfide to sulphydryl groups and activation of amino acids for protein synthesis.

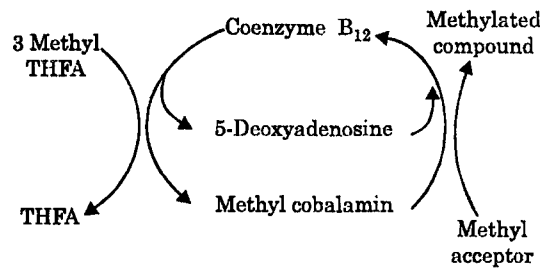


Fig. 16.14. Role of cobalamin in transmethylation reaction.

16.19 Vitamin C or Ascorbic Acid

Introduction : Vitamin C is more related to the monosaccharides than other vitamins.

Ascorbic acid is soluble in water but insoluble in fats and oils. It is one of the important biochemical redox systems, because it can get oxidized reversibly to dehydroascorbic acid. Chemically, it is a hexose derivative having empirical formula $C_6H_8O_6$.

Physiological functions : When ascorbic acid acts as a donor of reducing equivalents it is oxidized to dehydroascorbic acid, which itself can act as a source of the vitamin. Ascorbic acid is a reducing agent with a hydrogen potential of + 0.08 V, making it capable of reducing such compounds as molecular oxygen, nitrate, and cytochromes *a* and *c*. The mechanism of action of ascorbic acid in may of its activities is far from clear, but the following are some of the better documented processes requiring ascorbic acid.

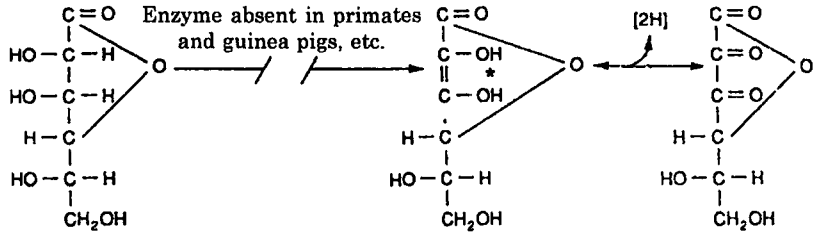


Fig. 16.15. Ascorbic acid, its source in nonprimates, and its oxidation to dehydroascorbic acid, ionizes in ascorbate.

1. Hydroxylation of proline in *collagen synthesis.*

2. In the *degradation of tyrosine,* the oxidation of *p*-hydroxyphenylpyruvate to homogentisate requires vitamin C, which may maintain the reduced state of copper necessary for maximal activity. The subsequent step is catalyzed by homogentisate dioxygenase, which is a ferrous iron-containing enzyme that also requires ascorbic acid.

3. In the *synthesis of epinephrine from tyrosine* at the dopamine β-hydroxylase step.

4. In *bile acid formation* at the initial 7α-hydroxylase step.

5. The *adrenal cortex* contains large amounts of vitamin C, which is rapidly depleted when the gland is stimulated by adrenocorticotropic hormone. The reason for this is obscure, but steroidogenesis involves several reductive syntheses.

6. The *absorption of iron* is significantly enhanced by the presence of vitamin C.

7. Ascorbic acid may act as a general water-soluble *antioxidant* and may inhibit the formation of nitrosamines during digestion.

Occurrence : Vitamin C is widely distributed in both plants and animals. In plants, it is mainly found in citrous fruits like lemons, oranges, black carrots, etc. It is also found in green vegetables like cabbage, beans and tomatoes. In animals, it occurs in tissues and various glands or organs (*e.g.*, liver adrenal glands, thymus, corpus luteum, etc.) In small quantities, it also occurs in milk and blood.

The structure of ascorbic acid (Fig. 16.15) is reminiscent of glucose, from which it is derived in the majority of mammals. However, in primates, including man, and a number of other animals, *e.g.*, guinea pigs, some bats, birds, fishes, and in vertebrates, the absence of the enzyme L-gulonolactone oxidase prevents this synthesis.

Scurvy is the classical syndrome of vitamin C deficiency. It is related to defective collagen synthesis which is indicated by subcutaneous and other hemorrhages, muscle weakness, soft swollen gums, and loose teeth; and is cured by consumption of fruits and fresh vegetables. The normal stores of vitamin C are sufficient to last 3-4 months before signs of scurvy appear.

Deficiency disease : The deficiency of vitamin C causes the disease scurvy (*i.e.*, tendency to haemorrhage and structural changes in the cartilage, bone and teeth) in infants and adults. In severe deficiency, there occurs swelling and bleeding of gums and the teeth become lose. This can also cause anaemia, lengthen the time for coagulation of blood and may cause the blood capillary to become fragile.

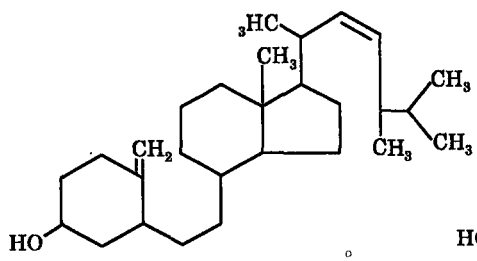
Most of the animals can synthesise vitamin C themselves and, therefore, they have not to depend upon the external supply. But human beings, monkeys and pigs are unable to synthesize this vitamin and hence they have to depend upon external supply.

16.20 Vitamin D

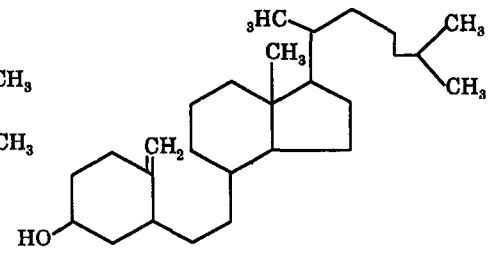
Introduction : Vitamin D represents a group of fat-soluble vitamins which are structurally related to sterols. Uptil now, five vitamins, *e.g.*, D_1, D_2, D_3, D_4 and D_5 have been isolated. In fact, vitamin D_1 has been found to be a molecular compound of D_2 and lumisterol.

All the types of vitamin D can be prepared from irradiation of the specific sterols called provitamins, *e.g.*, vitamin D_2 from ergosterol, vitamin D_3 from 7 dehydrocholesterol, vitamin D_4 from 22-23-dihydroergosterol and vitamin D_5 from 7-dehydro-sitosterol.

Vitamin D_2 is also called ergocalciferol.

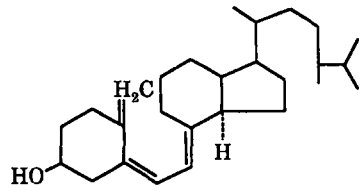


Vitamin D_2 (Ergocalciferol)

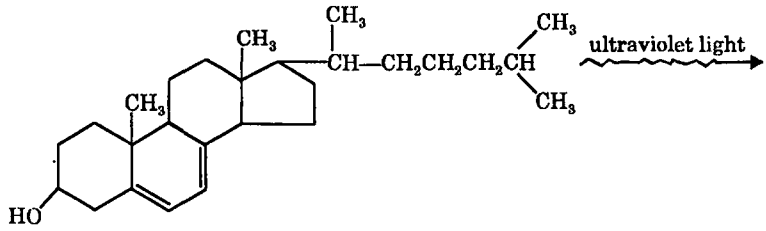


Vitamin D_3 (Cholecalciferol)

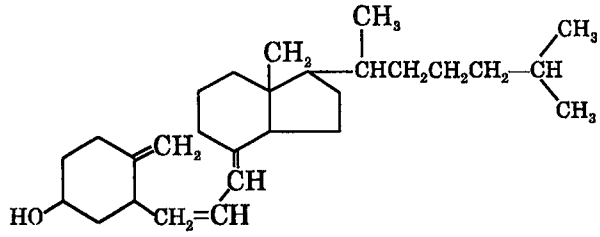
Occurrence : Vitamin D_2 is formed in nature by the irradiation of sterols. However, other sources are cod liver oil and other fresh liver oils, hen's eggs and milk of mammals. The amount of the vitamin D_2 in the liver of fish depends upon the season and also upon the biological conditions of the environment in which fish is living.



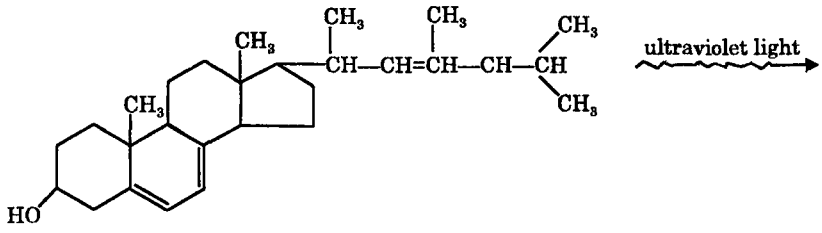
Vitamin D_4—22, 23-Dihydro-5, 6-cis-ergocalciferol



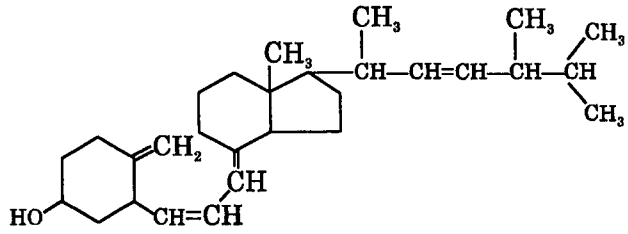
7-dehydrocholesterol



Vitamin D_3



Ergosterol



Vitamin D_2 (calciferol)

Vitamin D is sometimes called *the sunshine vitamin.* This is because 7-dehydrocholesterol, a steroid normally found in human skin, is converted to vitamin D_3 when irradiated by ultraviolet light. *Ergosterol,* found in molds and yeasts, is converted to vitamin D_2 (calciferol) when irradiated by sunlight. The natural vitamin D content of milk and other foods is supplemented by addition of irradiated ergosterol.

It has been determined that active vitamin D has two —OH groups. One is added in the liver, the second in the kidneys. Patients with kidney or liver damage are given a modified form of vitamin D, containing one —OH group.

Vitamin D increases Ca^{2+} and PO_4^{3-} absorption through the intestinal wall.

Deficiency disease : The deficiency of vitamin D_2 causes rickets which is a disease mainly occurring in children. This disease is characterised by the softening and bending of bones. But these conditions respond rapidly to vitamin D_2. Therefore, vitamin D_2 is known as *antirachitic factor.*

Rickets does not affect adults since their bone formation is completed. Because vitamin D is lipid soluble, it can be stored. This, coupled with excessive intake of the vitamin (hypervitaminosis), can cause bone demineralization (loss of Ca^{2+} from bone) and loss of bone strength. These can lead to multiple fractures even under mild strain. In *vitamin D hypervitaminosis,* blood serum levels of calcium and phosphate ions increase. This can cause calcium phosphate deposits in soft tissues and in the kidneys.

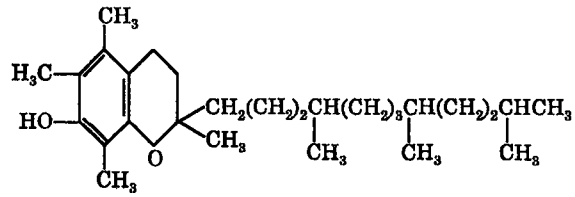
Physiological functions : The main function of D_2 is to maintain control of calcium and phosphorus metabolism. It increase both active as well as passive transport of calcium through small intestine. Vitamin D_2 also increases the volume of gastric secretion.

16.21 Vitamin E or Tocopherols

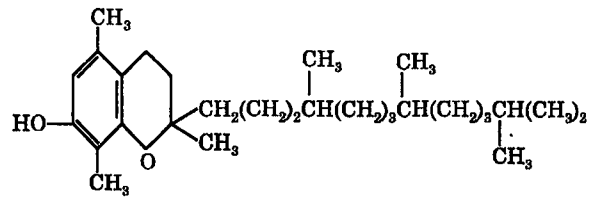
Introduction : Vitamin E represents a group of eight compounds which are collectively called *tocopherols.* These are α-, β-, γ-, δ-, ε-, ξ_1, ξ_2- and *n.* Out of these, most biologically active compound is α-tocopherol whereas β- and γ- tocopherols exhibit about half the activity of α-compound.

Vitamin E was discovered by Sure in 1922. The structures of α-, β- and γ- tocopherols are given as follows :

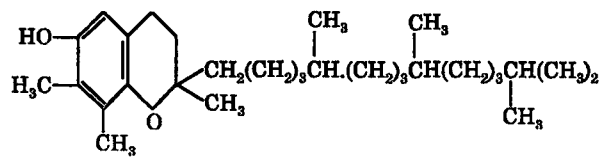
D-α-Tocopherol has the widest natural distribution and the greatest biological activity.



(±)-α-Tocopherol



(±)-β-Tocopherol



γ-Tocopherol

Active Fat Absorption Promotes the Absorption of Vitamin E

Impaired fat absorption leads to vitamin E deficiency because tocopherol is found dissolved in the fat of the diet and is liberated and absorbed during fat digestion. Furthermore, it is *transported in the blood by lipoproteins,* first, by incorporation into chylomicrons, which distribute the vitamin to the tissues containing lipoprotein lipase and the liver in chylomicron remnants, and second, by export from the liver is very low density lipoproteins. It is stored in adipose tissue.

Vitamin E is a Most Important Natural Antioxidant

Vitamin E appears to be the first line of defense against *peroxidation of polyunstaturated fatty acids* contained in cellular and subcellular membrane phospholipids. The phospholipids of mitochondria, endoplasmic reticulum, and plasma membranes possess affinities for α-tocopherol and the vitamin appears to concentrate at these sites. The tocopherols act as antioxidants, breaking free radical chain reactions as a result of their ability to transfer a phenolic hydrogen to a peroxyl free radical of a peroxidised polyunsaturated fatty acid. The phenoxy free radical formed then reacts with a further peroxyl free radical. Thus, α-tocopherol does not readily engage in reversible oxidation; the chromane ring and the side chain are oxidized to the non-free-radical product. This oxidation product is conjugated with glucuronic acid via the 2-hydroxyl group and excreted in the bile. Unlike some other vitamins, *e.g.,* niacin, B_{12}, and folate, tocopherol is not recycled after carrying out its function but most be replaced totally to continue its biological role in the cell. The antioxidant action of tocopherol is effective at high oxygen concentrations, and thus it is not surprising that is tends to be concentrated in those lipid structures that are exposed to the highest O_2 partial pressures, *e.g.,* the erythrocyte membrane and the membranes of the respiratory tree.

Vitamin E and Selenium Act Synergistically and Reduce the Body's Requirement for Each Other : *Glutathione peroxidase,* of which selenium is an integral component, provides a second line of defence against peroxides before they can propagate in chain reactions, damaging membranes and other cell components. Thus, tocopherol and selenium reduce each other's requirement or reinforce each other in their actions against lipid peroxides. In addition, selenium is required for normal pancreatic function, which is necessary for the digestion and absorption of lipids, including vitamin E. Conversely, vitamin E reduces selenium requirements by preventing loss of selenium from the body or maintaining it in active form.

Occurrence : It is widely distributed in nature in plant and animal tissues. This vitamin occurs in wheat germ oil (which contains α-and β-tocoperols), cotton seed oil (which contains α-tocopherol), soyabean oil (which contains δ-tocopherol), palm oil and rice. In animals, it occurs mainly in the livers of horses and cattles and in small amounts in the muscles of heart, kidney, placenta, egg. There is some evidence that all α-tocopherol is localised in the mitochondria.

Deficiency diseases : It is recommended to take 10-30 mg of vitamin E daily for an adult human being. Its deficiency in animals and man results in the following diseases :

(*i*) It causes antisterility. Due to this antisterility factor, the vitamin E was called tocopherol because word tokos (Greek) means childbirth and phero (Greek) means to bear.

(*ii*) It causes increases in the number of leucocytes, *i.e.,* WBC of the blood, causing blood anaemia.

(*iii*) It causes increased excretion of creative and pentose sugar (ribose) in urine which is primarily due to degeneration of muscles.

(*iv*) It also increases concentration of RNA and DNA in the bone marrow.

Physiological function : It is absorbed through intestine and this process is promoted by bile salts.

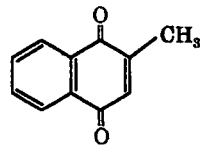
16.22 Vitamin K

Introduction : The term "*vitamins K*" was originally referred to the two compounds vitamin K_1 and K_2 which were related structurally but differed in both the length and the degree of unsaturation of an isoprenoid side-chain. Vitamin K_1 was first obtained in a pure state by Dam *et al.* (1939) and Doisy *et al.* (1939) from alfalfa and in the same year vitamin K_2 was isolated by Doisy *et al.* (1939) from putrefied fish meal. Subsequently vitamin K_2 was detected in a number of bacteria.

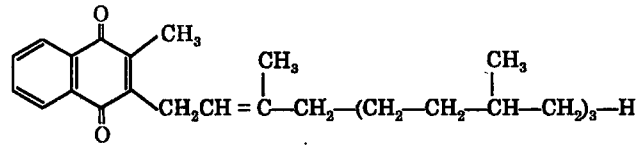
Vitamins belonging to the K group are poly-isoprenoid-substituted napthoquinones. Mendadione (K_3), the parent compound of the vitamin K series (Fig. 16.16), is not found naturally but if administered it is alkylated in vivo to one of the menaquinones (K_2). Phylloquinone (K_1) is the major form of vitamin K found in plants. Menaquionone-7 is one of the series of polyprenoid unsaturated forms of vitamin K found in animal tissues and synthsized by bacteria in the intestine.

Occurrence : Vitamin K_1 occurs in all green, leafy tissues, but the best sources are alfalfa, carrot tops, cabbage and spinach. Vitamin K_2 occurs principally in bacteria and also in putrefied fish meal.

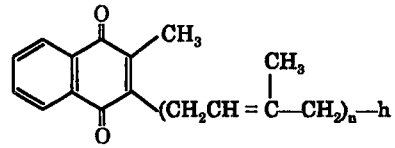
Properties : Vitamin K_1 is a yellow oil whereas vitamin K_2 is a yellow crystalline solid (m.p. 54°C). Both are sensitive to alkali and to light but are stable to heat. They exhibit characteristic and similar absorption spectra having maxima at 243, 249, 260, 270 nm (all with ε ~20,000) and 325 nm (ε ~ 3,000). Both the vitamins exhibit bands due to the presence of chromophore (common to both these vitamins) called 2, 3-disubstituted, 1, 4 naphthaquinone.



Menadione (vitamin K_3)



Phylloquinone (vitamin K_1, phytonadione, Mephyton)



Menaquinone-n (vitamin K_2; n = 6, 7, or 9)

Fig. 16.16. The naturally occurring vitamins K.

Deficiency disease : The vitamins K_1 and K_2 are required by man. The average daily requirement for man is almost 1 mg. Pregnant women are given vitamin K in order to check undue haemorrhage in both mother and child. Both are antihaemorrhagic vitamins because they are connected with the enzymes which are involved in blood clotting.Vitamins K_1 and K_2 are helpful in blood coagulation by activating prothrombin, the precursor of thrombin, for the formation of blood-clotting enzyme. The deficiency of vitamins K_1 and K_2 lengthens the time of blood clotting.

Hemorrhagic Disease of the Newborn is Caused by Deficiency of Vitamin K

Vitamin K is widely distributed in plant and animal tissues used as food, and production of the vitamin by the microflora of the intestine virtually ensures that dietary deficiency does not occur in adults. However, newborn infants are vulnerable to the deficiency, because the placenta does not pass the vitamin to the fetus efficiently and the gut is sterile immediately after birth. In normal infants, the plasma concentration decreases immediately after birth but recovers after food is absorbed. If the prothrombin level drops too low, the hemorrhagic syndrome may appear.

Vitamin K deficiency can be caused by fat malabsorption, which may be associated with pancreatic dysfunction, biliary disease, atrophy of the intestinal muscosa, or any cause of steatorrhea. In addition, *sterilization of the large intestine by antibiotics* can result in deficiency when dietary intake is limited.

Physiological functions : These vitamins K_1 and K_2 are absorbed in the intestine. Their absorption gets hastened in the presence of bile salts. Although vitamins K_1 and K_2 are stored but the site of their storage is not known with certainty.

Absorption of vitamin K requires normal fat absorption : Fat malabsorption is the commonest cause of vitamin K deficiency. The naturally occurring K derivatives are absorbed only in the presence of bile salts, like other lipids, and are distributed in the bloodstream via the lymphatics, in chylomicrons. Menadione, being water soluble, is absorbed *even in the absence of bile salts,* passing directly into the hepatic portal vein. Although vitamin K accumulates initially in the liver, its hepatic concentration declines rapidly and storage is limited.

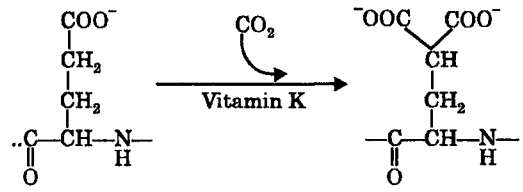


Fig. 16.17. Carboxylation of a glutamate residue catalyzed by vitamin K.

Vitamin K is Required for the Biosynthesis of Blood Clotting Factors

Vitamin K has been shown to be involved in the maintenance of normal levels of *blood clotting factors* II, VII, IX and X, all of which are synthesized in the liver initially as inactive precursor proteins.

Vitamin K Acts as cofactor of the carboxylase that forms γ-carboxyglutamate residues in precursor proteins : Generation of the biologically active clotting factors involves the *posttranslational modification* of glutamate (Glu) residues of the precursor proteins to γ-carboxyglutamate (Gla) residues by a specific vitamin K-dependent carboxylase (Fig. 16.17). Prothrombin (factor II) contains 10 of these residues, which allow *chelation of calcium* in a specific protein-calcium-phospholipid interaction, essential to their biologic role. Other proteins containing K-dependent Gla residues have now been identified in several tissues.

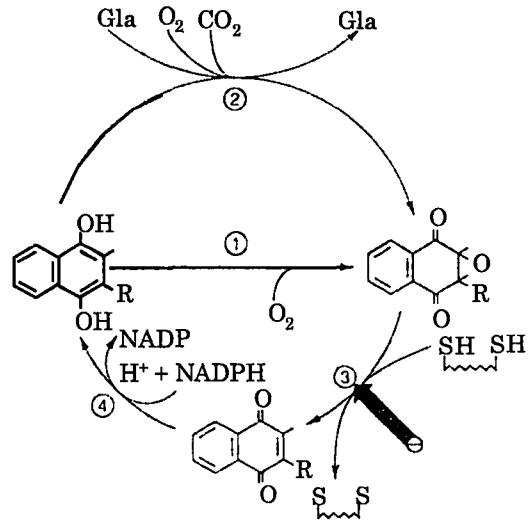


Fig. 16.18. Vitamin K-related metabolic activities in liver. The locus of action of the dicumarol-type anticoagulants is indicated. The details of some of the reactions are still uncertain. 1, monoxygenase; 2, carboxylase; 3, 2, 3-epoxide reductase; 4, reductase.

Vitamin K Cycle Allows Reduced Vitamin K to be Regenerated

The vitamin K-dependent carboxylase reaction occurs in the endoplasmic reticulum of many tissues and requires molecular oxygen, carbon dioxide (not HCO_3^-), and the *hydroquinone* (reduced) form of vitamin K. In the endoplasmic reticulum of liver there exists a *vitamin K cycle* (Fig. 16.18) in which the 2, 3-*epoxide* product of the carboxylation reaction is converted by 2, 3-epoxide

reductase to the quinone form of vitamin K, using an, as yet, unidentified dithol reductant. This reaction is *sensitive to inhibition by 4-hydroxydicoumarin (dicumarol) types of anticoagulant,* such as warfarin (Fig. 16.19). Subsequent reduction of the quinone form to the hydroquinone by NADH completes the vitamin K cycle for regenerating the active form of the vitamin.

An important therapeutic use of vitamin K is as an antidote to poisoning by dicumarol-type drugs. The quinone forms of vitamin K will bypass the inhibited epoxide reductase and provide a potential source of the active hydroquinone form of vitamin K.

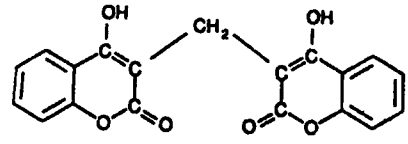


Fig. 16.19. Dicumarol (bishydroxycoumarin, 3, 3'-methylene-bishydroxycoumarin)

17 Metabolism of Carbohydrates

17.1 Introduction

Metabolism of carbohydrates is described under the following two headings :

1. Photosynthesis in green plants.
2. Biological oxidation of carbohydrates.

17.2 Photosynthesis in Green Plants

Monosaccharides are formed in green plants by photosynthesis or in other organisms by the reversal of glycolytic pathway.

17.3 Biosynthesis of Carbohydrates in Photosynthesis

Introduction : The energy which supports the activities of most living organisms on the earth is derived directly or indirectly from the energy of sunlight through photosynthesis (*Photo* = light; *synthesis* = to build up).

The usual way of defining photosynthesis may be as follows :

"It is the process in which simple carbohydrates are synthesised from water and carbon dioxide in the chlorophyll containing tissues of plants in the presence of sunlight; oxygen being a by-product is given out."

According to Kamen (1963), photosynthesis may be defined as follows :

"It is a series of processes in which electromagnetic energy is converted into chemical free energy which can be used for biosynthesis."

By a single over-all reaction, photosynthesis may be represented as follows :

$$CO_2 + H_2O \xrightarrow[\text{Chlorophyll}]{h\nu} 1/6\ (C_6H_{12}O_6) + O_2$$

In more general way, it may be

$$CO_2 + H_2O \xrightarrow[\text{Chlorophyll}]{h\nu} 1/n(CH_2O)n + O_2$$

The released oxygen (O_2) in photosynthesis comes from water and not from CO_2. It has been proved by using radioactive water [H_2O^{18}]. When green plants were supplied with water containing [H_2O^{18}], the released oxygen was entirely of the O^{18} types, confirming that water is only source to release the oxygen in photosynthesis. This can be represented by the following summary reaction :

$$6CO_2 + 12H_2O^{18} \longrightarrow C_6H_{12}O_6 + 6H_2O + 6O_2{}^{18}$$

Mechanism of photosynthesis : Many theories were propounded by various workers. But the earlier theories failed and still no theory is available which describes the mechanism satisfactorily. The uncertainty in the mechanism is due to the following reasons :

(*i*) No products of the earlier stages of the process could be detected experimentally.

(*ii*) When chlorophyll is extracted from plant, it fails to reproduce its photosynthetic property. So the photosynthesis cannot be studied under controlled experimental conditions.

(*iii*) The complete structure of the chlorophyll was not known fully.

Enough evidence has accumulated in the recent past to show that photosynthesis involves two states : a light phase and a dark phase.

The reaction of light phase is light sensitive and is therefore called a photochemical reaction.

The reactions of the dark phase do not require light and are therefore called temperature sensitive. Such reactions are also known as Blackman reactions.

$$\xrightarrow[\text{Photochemical reactions}]{\text{Step I}} \xrightarrow[\text{Chemical reactions}]{\text{Step II}} \text{Photosynthetic product}$$

We will discuss the light phase and dark phase reactions separately.

1. Light phase

Evidence in support of light phase : Hill and Scurisbrick found that isolated chloroplasts when illuminated produced oxygen from water. If certain hydrogen acceptors (oxidants) were present in the medium, they were reduced by the chloroplasts. The oxidants are called Hill reagents and the reactions as Hill reactions. Hill attributed the production of oxygen and hydrogen from water by light. Arnon in 1954, working on isolated chloroplasts, reported that in addition to carrying out the Hill reaction, the chloroplasts could also synthesis ATP in the light from ADP and inorganic phosphate, Pi. This photosynthetic phosphorylation may be represented as follows :

$$ADP + Pi \xrightarrow{\text{Light energy}} ATP$$

Nature of Light Energy : The energy for photosynthesis is derived from light. Although sunlight appears white it is actually a mixture of different colours. The *electromagnetic spectrum* consists of radiations of different wavelengths (17.1). It includes gamma rays, X-ray, ultraviolet rays, visible spectrum, infrared rays and radio waves. Each of these types of waves has a characteristic range of wavelengths. The *visible spectrum* is constituted by the bands of colours

visible to the human eye. It ranges from 3,800-7,800A, and constitutes just a small region of the spectrum of electromagnetic radiations.

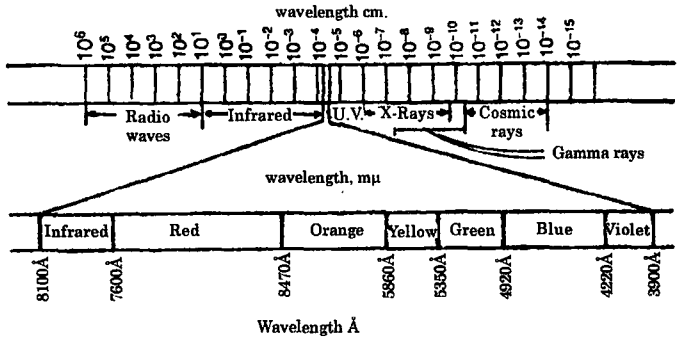


Fig. 17.1. The electromagnetic spectrum.

According to the *wave theory* of light, each colour of the spectrum consists of a different wavelength. Red light at one end of the spectrum has a longer wavelength than *violet light* at the other end. The longer the wavelength the less the energy conveyed. Conversely, shorter wavelengths convey more energy. Thus, red light with a longer wavelength than violet conveys less energy than violet light.

According to the second model of the duality of light, it is held that light is composed of a stream of tiny particles called *quanta* or *protons*. The theory is known as the *particulate or quantum* theory. The photons or transmission particles are discrete units or packets of light energy.

Absorption spectrum : The amount of absorption of light at different wavelengths constitutes the *absorption spectrum*. If light of different wavelengths is passed through a chlorophyll extract it is possible to measure the absorption at each wavelength. An absorption spectrum of chlorophyll *a*, the main light absorption pigments shows that light is mainly absorbed in the blue and red regions. Green, yellow and orange light is absorbed only slightly. This is to be expected because chlorophyll solutions are green, and therefore do not absorb green light but select it.

The relationship between different colours of light and photosynthesis was established by Von Engelmann in 1882 (Fig. 17.1 A). Filaments of the green alga *Spirogyra* were placed on a slide. They were illuminated through a prism with a spectrum of light. A drop of water containing hundreds of aerobic bacteria was placed on the algal filaments. These bacteria depend upon oxygen for respiration. It was found that after a few minutes the bacteria clustered around the filament and were concentrated in the blue and red regions. It was in those regions of light that maximum oxygen was available, indicating maximum photosynthetic activity.

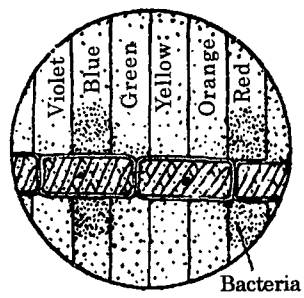


Fig. 17.1 (A). Von Englemann's experiment showing clustering of aerobic bacteria around a filament of Spirogyra in the blue and red regions of the spectrum.

Transmission, absorption and reflection of light (Fig. 17.1 B). When light falls on a leaf it either passes through the leaf (*i.e.*, is *transmitted*), or is *absorbed* or *reflected*. In general, leaves transmit 5% light, reflect 12% and absorb 83%. Of the absorbed light only 4% is absorbed by chlorophyll, the remaining 79% being lost as heat.

Excitation of electrons : As mentioned previously, light comes in the form of packets or *photons*. When an atom absorbs a photon it is said to be in '*activated*' or '*excited*' state. Such excitation creates an unstable condition within the molecule. This unstability is very brief ($10^{-8/9}$ second), and the excited electrons return to their normal energy level. All atoms contain electrons which travel around the nucleus in one or more orbits. The electrons of the outermost orbit are the most chemically reactive, since they are the most weakly bound to the nucleus.

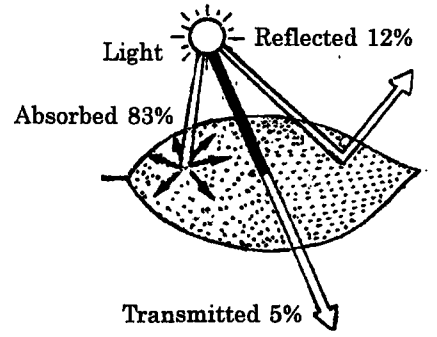


Fig. 17.1 (B). Transmission, absorption and reflection of light falling on a leaf.

Light energy absorbed by chlorophyll can be released as *flourescence, phosphorescence* or *heat* (waste), or can be utilized for useful work. When a photon of light is absorbed by an atom in the *low energy state* (ground state), the electron is pushed into a *high energy orbit* (Fig. 17.1 C and 17.1 D). This orbit is further away from the nucleus. The electron therefore becomes *unstable* or *excited* and is said to be in the *singlet* state. It can exist in this stage for only about 10^{-9} second. The electron now behaves in one of the following ways : (1) It can return to the low energy ground state, releasing the absorbed energy. This emission of radiant energy in visible form is called *flourescence*. (2) The excited high-energy electron can drop into a slightly lower energy state called the *triplet state*. It can exist in the triplet state for 10^{-3} second, *i.e.*, much longer than in the singlet state. (3) The electron can further drop from the triplet state back to the ground state. Here also energy emission in visible form takes place, and is called *phosphorescence*. (4) The electron can also drop from the excited state to the ground state with the release of *heat*.

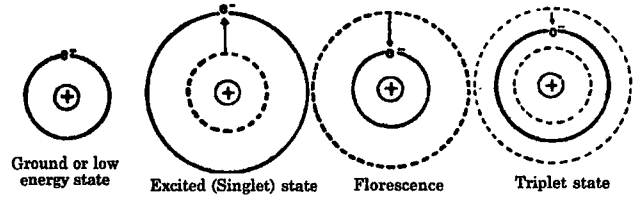


Fig. 17.1 (C). Photochemical changes in chlorophyll.

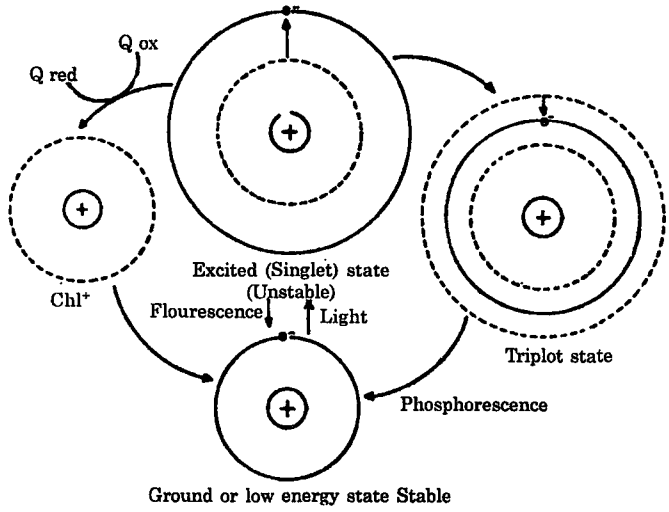


Fig. 17.1 (D). Electron orbits in different energy states of chlorophyll.

When a photon of light strikes a chlorophyll molecule, it can transfer its energy to the outer electron. This electron now becomes excited and is raised to higher energy level. The molecule then becomes unstable. The excited electron is now trapped by various electron acceptors. Chlorophyll thus converts light energy of photons into chemical-bond energy which is stored in ATP. It has been found that chlorophyll in a test tube immediately loses the trapped energy from photons, and emits it as visible light.

Photosynthesis Apparatus

Photosynthesis takes place within the *plastids* except in blue green algae, and bacteria. In green plants the plastids contain *chlorophyll,* and are called *chloroplasts.* Chloroplasts contain many membranous bodies called *grana.* Each granum consists of disc-shaped membranous sacs called *thylakoids* piled one on top of the other like coins.

The lamellae of the discs of spinach chloroplasts have arrays of repeating subunits called *quantasomes.* These are believed to be the units of photosynthesis. The quantasomes are ellipsoid plates about 200 A in diameter and 100 A thick. They are arranged in a regular pattern on the membranes of the grana. In spinach quantasomes there are about 160 molecules of *chlorophyll a, 70* of *chlorophyll b* and several molecules of other substances.

In photosynthetic bacteria all the pigments and enzymes necessary for photosynthesis are located in *chromatophores.* These are roughly spherical particles, about 300 A in diameter, which are attached to the cell membrane or may be an integral part of the membrane.

Photosynthesis Pigments

In higher plants and green algae the chlorophyll actually consists of two closely related pigments, *chlorophyll a (Chl a)* and *chlorophyll b (Chl b)* (Fig. 17.1 E). Chlorophyll *a* is the principal pigment and chlorophyll *b* the accessory pigment. In brown algae, diatoms and flagellates, chlorophyll *a* is the principal pigment and chlorophyll *c* the accessory pigment. Red algae contain chlorophyll *a* as the principal pigment and chlorophyll *d* as the accessory pigment. In bacteria the most important photosynthetic pigment is *bacteriochlorophyll* (B Chl). Green sulphur bacteria contains *chlorobium chlorophyll* as the principal pigment, plus traces of B Chl.

In addition to the pigments mentioned above other accessory pigments may be present. These include *carotenoids* and *phycolobins* (*phycocyanin*) and *phycoerethrin* (in red algae).

Structure of chlorophyll : The empirical formula of chlorophyll *a* is $C_{55}H_{72}O_5N_4Mg$. Chlorophyll *a* is a blue-green microcrystalline solid, consisting of *head* and a *tail.* The head consists of a *porphyprin ring* or *tetrapyrrole nucleus* from which extends a tail made up of a 20 carbon grouping called the *phytol.* The *porphyrins* are complex carbon nitrogen molecules that usually surround a metal. In *chlorophyll* the porphyrin surrounds a *magnesium* while in *haemoglobin* surrounds an *iron.* The cytochromes of the electron transport system also have porphyrin rings. The basic unit of the porphyrin ring is the *porophobilinogen* molecule. Four such *pyrrole* units make up the *tetrapyrrole* structure. *Phytol* is a along straight chain alcohol containing a single double bond. It may be regarded as a hydrogenated carotene (vitamin A). Its formula is $C_{20}H_{39}$.

Chlorophyll *b* has the empirical formula $C_{55}H_{70}O_6N_4Mg$. It is a green-black microcrystalline solid, it differs from chlorophyll *a* in having *aldehyde* (CHO) group attached to carbon atom 3, instead of a *methyl* (CH_3) group.

Absorption spectrum of chlorophyll (17.1 F). As mentioned previously the amount of light (energy) absorbed at different wave lengths constitutes the *absorption spectrum.* It has also been

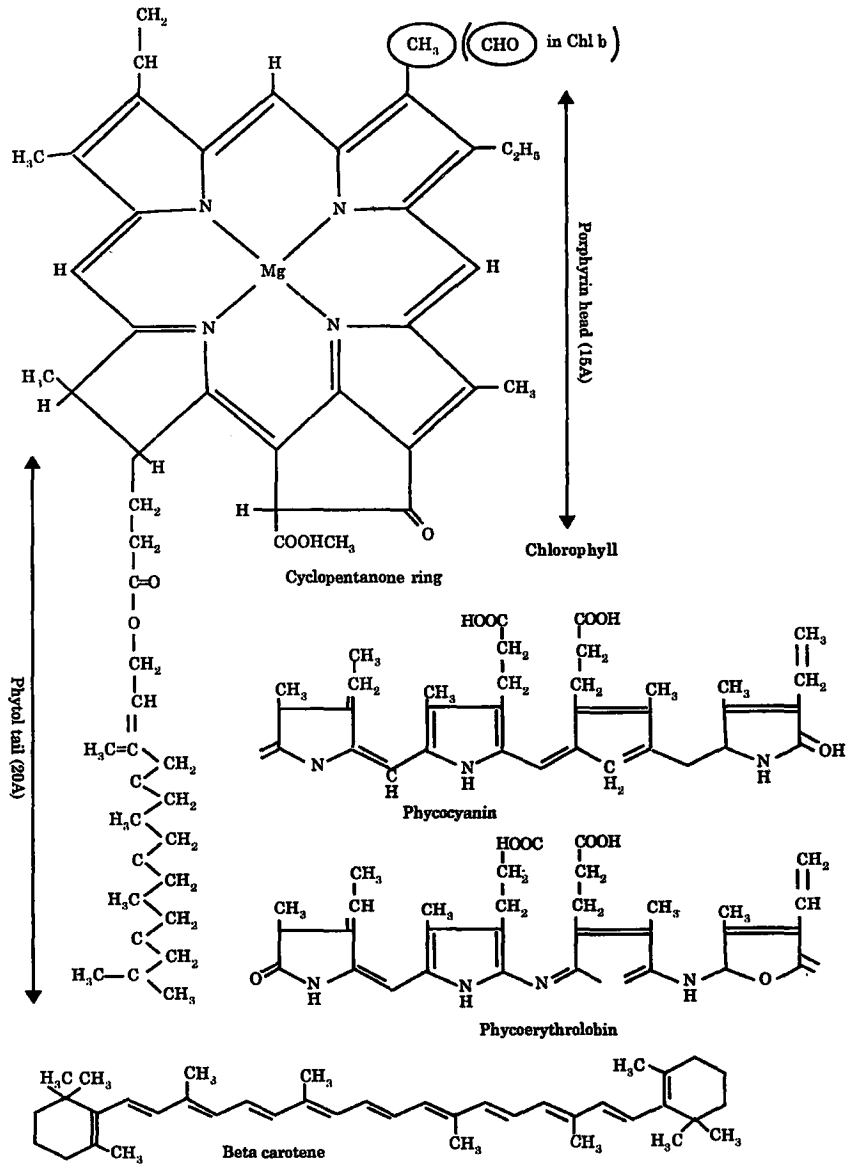


Fig. 17.1 (E). Photosynthesis pigments : Chlorophyll *a* and *b* phycocyanin, phycoerythrolobin and β- carotene.

seen that the clorophyll of higher plants absorbs mainly in the blue and red regions of the spectrum. There is some difference in the absorption spectra of chlorophyll *a* and chlorophyll *b*. Chlorophyll *a* has a major absorption peak at 675 mμ and a minor peak at 450 mμ. The major absorption spectrum for chlorophyll *b* is at 450 mμ. Recent work has shown that extraction procedures change the absorption properties of chlorophyll. Thus, the absorption spectra for extracted chlorophyll and chlorophyll *in situ* are not same. *In situ* chlorophyll *a* absorbs mainly far redlight (700 mμ) while chlorophyll *b* primarily absorbs lower wavelengths of light.

Bacteriochlorophyll absorbs in the infra-red and blue-violet regions.

Action spectrum : (Fig. 17.1 G). A study of the absorption spectra shows that light is primarily absorbed in the blue and red regions. Green, yellow and orange light is absorbed only

slightly. If the relative effectiveness of photosynthesis at different wavelengths (*action spectrum* of photosynthesis) is, however, studied, it is found that it differs from the absorption spectrum. There is quite a lot of photosynthetic activity even in parts of the spectrum where chlorophyll *a* absorbs little light. This suggests that the light energy absorbed by other pigments (mainly yellow and orange carotenoids, and also other forms of chlorophyll) is transferred to chlorophyll *a*.

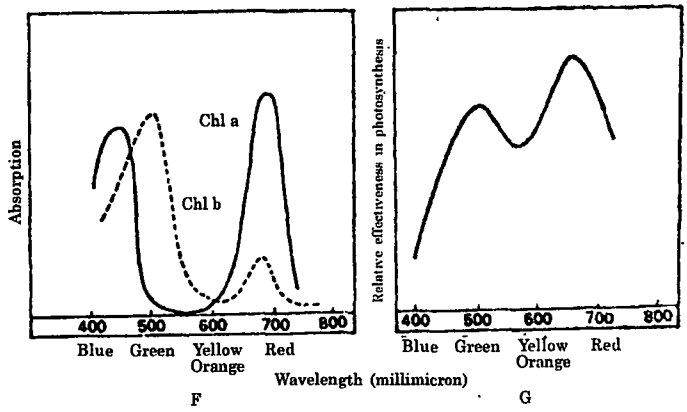
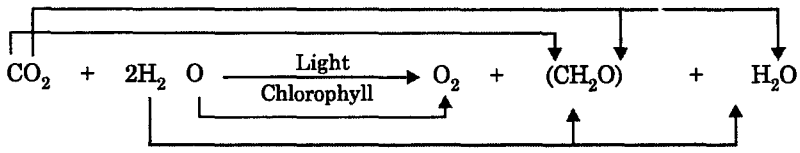


Fig. 17.1 (F). Absorption spectra for chlorophylls *a* and *b*.
Fig. 17.1 (G). Action spectrum of photosynthesis.

Mechanism of Photosynthesis

The basic equation for photosynthesis in green plants is :



The arrows in the above equation indicate the fate of the atoms.

If this equation is multiplied by 6, the formation of *glucose,* a hexose (6-caroon) sugar is indicated.

$$6CO_2 + 12H_2O \xrightarrow[\text{Chlorophyll}]{\text{Light}} 6O_2 + C_6H_{12}O_6 + 6H_3O$$

It will be seen that in the above equation water has been shown on both sides. This is necessary because the water produced in photosynthesis is new water, and not the same water which is used as raw material.

(A) Outline of Photosynthetic Reactions

There are two reactions involved in photosynthesis. The first reaction requires light and is called the *light* or *Hill reaction.* The second reaction does not require light and is called the *dark* or *Blackman reaction.* (1) The *light reaction* is a photochemical reaction, while the dark reaction is a *thermochemical* reaction. The unit of photosynthesis is believed to consist of two types of centres, *photosystem I* and *photosystem II.* These are excited at different wavelengths of light. The two systems are linked by redox catalysts. The light reaction involves two processes, *photophosphorylation* and *'photolysis' of water.* In photophosphorylation there is conversion of light energy into chemical energy. *Photophosphorylation* is of two types, *cyclic photophosphorylation* and *noncyclic photophosphorylation.* (2) The *dark reaction* takes place through a series of steps known as the *Calvin-Benson Cycle.* The details of different stages of photosynthesis will now be taken up.

(B) The Light or Hill Reaction

The first photosynthetic reaction taking place in the presence of light is called the *light or Hill reaction.* The light reaction involves two porcesses, *photophosphorylation* and *'photolysis'*

of water. The essential features of the light reaction are : (1) the absorption of light energy by chlorophyll, (2) the transfer of this energy and (3) the utilization of the energy in the electron transfer chain. The process by which light energy is transformed into chemical energy is called *photophosphorylation*. There are two types of photophorylation, *cyclic* and *noncyclic photophosphorylation*.

(1) Photosytems I and II

In higher plants and algae, two pigment systems are involved in photophosphorylation. These are called *photosystems I and II*. The pigments of the two systems are known as *pigment system I (PS I)* and *pigment system II (PS H), respectively*. PS I and PS II are structurally distinct.

PS I and PS II both contain *chlorophyll a, chlorophyll b* and *carotenoids*. The distribution of the two pigments, however, varies in the two systems. PS I contains more *carotenes* than PS II. *Xanthophyll* predominates in PS II. The primary photosynthetic pigment of both systems is chlorophyll *a*. In blue-green and red algae *phycobiliproteins* (formerly *phycoerythrin* and *phycocyanin*) are present as accessory pigments.

Gathering of light : reaction centres : The reaction centre is considered to be a special chlorophyll complex associated with a primary electron donor (D) and acceptor (E). When excited by light photons the following reaction sequence is presumed to occur :

$$DChlA \xrightarrow{h\nu} DChl^*A \longrightarrow DChl^+A^- \longrightarrow D^+ChlA^-$$

Each photosystem contains many chlorophyll molecules. It is believed that most of the chlorophyll molecules of a photosystem are *'antenna'* or *'light harvesting'* molecules (Fig. 17.1 H). One chlorophyll molecule, however is the *reaction centre*. Energy conversion can take place only in this molecule. The antenna chlorophyll molecules serve only to absorb light photons. A photon either moves about at random around the chlorophyll molecule until it is lost as heat or flourescence, or is trapped by the reaction centre molecule. If magnesium atoms are removed from the antenna chlorophyll molecules of photosynthetic bacteria the photosynthetic unit continues to harvest light photons for the reaction centre. However, if magnesium is removed from the reaction centre molecule photosynthesis stops. The trapping effect of the reaction centre is due to the fact that it has a lower energy level than the other chlorophyll molecules.

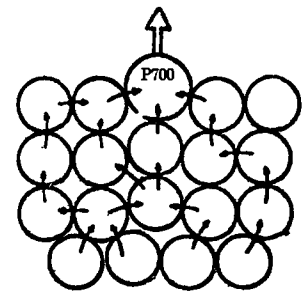


Fig. 17.1 (H). Antenna chlorophyll molecules and the reaction centre.

The antenna complex consists of several hundred chlorophyll molecules and other pigments bound to proteins. There are about 300 primary absorbing or antenna chlorophyll molecules for every reactive molecule. The reaction centre of PSI absorbs light maximally at 700 nm and is therefore called P700. The PS II reaction centre absorbs maximally at around 680 nm and is called P680. In each photosystem the energy of excitation is conveyed from the antenna chlorophyll molecule to the reaction centre molecule. Light energy brings about a change in the distribution of electrons in the ring of the chlorophyll molecule and releases one electron for transfer. This process is repeated on absorption of another photon. Each photon absorbed at a centre causes the excited chlorophyll molecule (Ch^*) to donate one electron to an acceptor (A).

$$Chl \xrightarrow{h\nu} Chl^* \xrightarrow{A} Chl^+ + A^-$$

It is now generally accepted that the reaction centre chlorophyll exists in the form of a *dimer.*

When the chloroplast membrane is subjected to disc-gel electrophoresis two protein bands containing chlorophyll are observed. These represent the two chlorophyll protein complexes (CP I and CP II). Chlorophyll-protein complex I contains 28% of the total membrane protein, while chlorophyll-protein complex II contains 50%. Chlorophyll *a* is found in both complexes, while chlorophyll *b* is found only in complex II. The proteins in the chlorophyll-protein complexes probably maintain chlorophyll molecules in an orientation where maximal harvesting of light can occur. The antenna chlorophyll *a* molecules of PS I and PS II are probably bound to different proteins, or are embedded in different matrices. Light energy is probably transferred by inductive resonance from the accessory pigments to the reaction centre in the two pigment systems. In eukaryotic cells the pathway is as follows :

Light energy ⟶ *Carotenoids* ⟶ *Chlorophyll a* ⟶ *Reaction centre*
↑
Chlorophyll b

The excitation energy of chlorophyll *b* is directly transferred to chlorophyll *a*. In PS II of blue green 'algae' and eukaryotic red algae, the pathway is as follows :

Carotenoids ⟶ Phycobiliproteins (Phycoeruthrin,- Phycocyanin) ⟶ Chlorophyll *a* ⟶ Reaction centre
↑
Chlorophyll *b*

Interaction of PS I and PS II : PS I is excited by light of wavelength shorter than 700 nm. It generates a strong reductant leading to the formation of NADPH via reduced ferredoxin. PS II is excited by light of shorter wavelength than 680 nm. It generates a strong oxidant that splits water and leads to the formation of O_2. In addition PS I produces a weak oxidant and PS II a weak reductant, the interaction of which leads to the formation of ATP (*photophosphorylation*).

Light ($\lambda < 700$ nm) ⟶ PS I { strong reductant → NADPH ; weak oxidant } → ATP

Light ($\lambda < 680$ nm) ⟶ PS II { weak reductant } → ATP ; strong oxidant → O_2

(2) Electron Transfer Components (Fig. 17.1)

Electron transport in the two photosystems takes place in three segments : (1) from H_2O to P 680, (2) from P 680 to P 700 and (3) from P 700 to NADPH. The electron transport system zig-zags across the thylakoid membrane from water to NADPH.

(i) Mn containing system : The enzymatic system mediating the flow of electrons from water to P680 contains Mn as an active component of the oxygen evolving system. The Mn appears to be associated with a protein and seems to be located in two pools of about two and four Mn per oxygen centre. Electrons are transported from water to P 680 of PS II through the Mn containing system. The ultimate source of electrons within the chloroplasts is water.

One molecule of oxygen is evolved for every four electrons donated to P680. These four electrons are formed by oxidation of two molecules of water.

$$2H_2O \longrightarrow 4H^+ + O_2 + 4e^-$$

The conversion of CO_2 to glucose during photosynthesis requires two molecules of NADPH

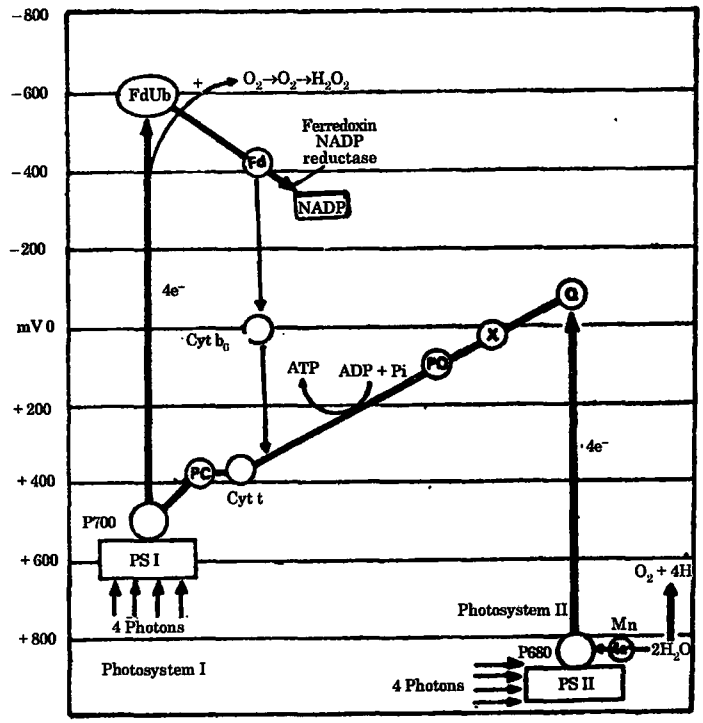


Fig. 17.1 (I). Diagrams of the two photosystems (I and II) and the connecting electron transfer chain (electron carriers).

per carbon atom converted, *i.e.*, two molecules of NADPH are required to reduce one molecule of CO_2.

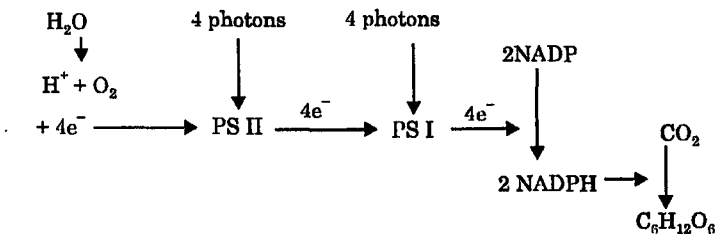
$$6CO_2 + 12(NADPH + H^+) \longrightarrow C_6H_{12}O_6 + 6H_2O$$

Two electrons are required for the reduction of each molecule of NADP to NADPH, *i.e.*, four electrons for the reduction of 2 NADP to 2NADPH.

$$2NADP + 2H_2O + 8\,h\nu \longrightarrow 2NADPH + O_2 + 2H^+$$

Two quanta of light (photons) are required for the passage of one electron through the electron transport system from water to NADP. One photon is necessary for electron transfer from H_2O to PS I and one for transfer PS I to NADP.

Thus in all four quanta are required for each NADPH formed and eight quanta for each CO_2 incorporated into carbohydrate. One photon of light is absorbed at PS II and one at PS I for each electron transferred from H_2O to NADP.



Dissociation of each water molecule results in the formation of two protons (H^+), two electrons (e^-) and oxygen.

$$H_2O \longrightarrow 2H^+ + 2e^- + 1/2\,O_2$$

The two electrons reduce two P680 molecules. The reaction is catalysed by an enzyme containing Mn. There is strong evidence that the 2-4Mn^{2+} ions found in each reaction centre are involved as electron donors to PS II. The two protons go into solution on the inner side of the inner membrane. The oxygen atom from the H_2O molecule diffuses out of the chloroplast.

(ii) Photosystem II (PS II) : The redox potential for P680 must be above 850mV (Blankenship and Parson, 1978). The antenna chlorophyll molecules absorb light photons and communicate the energy of excitation to the P680 reaction centre. The chlorophyll molecule has a ring like structure with a central magnesium atom. Light energy alters the distribution of electrons in P680 and one electron is released per photon absorbed. After releasing the electron P 680 becomes positively charged (P^+ 680). It can now accept one of the electrons released by the dissociation of H_2O.

(iii) Compound Q : The electron acceptor molecule of PS II is not known with certainty. It was originally called Q because it quenches (extinguishes) flourescence produced by illuminating P 680 of photo-system II. Q is not to be confused with coenzyme Q, which is also abbreviated as Q. A unique feature of PSII is that it produces variable flourescence on excitation. It has been shown that under anaerobic conditions Q is present as two components with potential levels of –35 mV and –270 mV, each carrying one electron. It was supposed that this effect was due to the two states of reduction of Q, *i.e.,* QH and QH_2. Knaff (1975) has suggested that the true $E^{0'}$ of the primary acceptor is about —130 mV. Most investigators believe that Q is one of the plastoquinones. In spinach chloroplasts plastoquinone A is the predominant form. In addition there are six other plastoquinones.

Excitation of PS II results in a UV absorbance peak at 320 nm (X 320). X 320, like Q, is inhibited by DCMU. It has been suggested that X 320 is associated with the primary acceptor of PS II and that it may be a form of plastoquinone. Stiehl and Witt (1969) have suggested that X 320 may be produced by reduction of PQ to the semiquinone anion.

The sequence of carriers between PS II and PS I is generally written as follows :

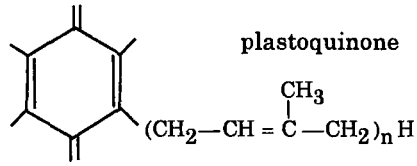
$$Q \longrightarrow PQ \longrightarrow \text{Cyt } b\ 559 \longrightarrow \text{Cyt } f \longrightarrow PC$$

The location of Cyt *b* 559 in the electron transport chain connecting PS II and PS I has been questioned.

The sequence PQ ⟶ Cyt *b* 559 ⟶ Cyt *f* is closely parallel to the UQ ⟶ Cyt b_T ⟶ Cyt c_i sequence of mitochondria, which includes the coupling site II for ATP synthesis. In chloroplasts also ATP synthesis is coupled to electron transport.

(iv) Plastoquinone (PQ) : The two electrons released from P680 are accepted by plastoquinone (PQ). The electrons, along with two protons extracted from the external solution, reduce PQ to PQH_2. PQH_2 migrates across the membrane to the inner surface and transfers the electrons to cytochrome *f*. The two protons are released internally.

It has been suggested that a pool of 10 to 20 equivalent of plastoquinone accepts electrons from Q. PQ is similar in structure to coenzyme Q. Several analogues of PQ have been found. PQ A with a 20 carbon side chain has been found in *Aesuclus*. PQ B and PQC have been found to occur in all the higher plants and algae (except *Anacystis*) as a series of six isomers. PQ is replaced by CoQ, menaquinone (MK) or chlorobium quinone in different photosynthetic bacteria.



(*v*) *Cytochrome b 559 :* The function of cyt *b* 559 in photosynthesis is not clear. Its redox potential was reported to be + 370 mV by Bendall (1968). Subsequent values fall into two groups centering around + 350 mV and + 70 mV. Wada and Arnon (1971) suggested that cyt *b* 559 could exist in three forms of differing redox potentials. It was thought that a high potential form (cyt *b* 559 HP) could be converted to lower potential forms by disruptive treatment. There is at least one, and possibly two, molecules of cyt *b* 559 in a photosynthetic unit of PS II.

It has been suggested that cyt *b* 559 HP exists in two pools, one of which is required for the evolution of O_2. However, other workers have reported that the oxygen-evolving system operates even in the absence of cyt *b* 559 HP. The bulk of the evidence indicates that cyt *b* 559 HP does not directly take part in the evolution of oxygen. The fact that cyt *b* 559 is photo-oxidized by PS II strongly suggests that it is in close association with the primary oxidant P680.

Several laboratories have reported the photo-oxidation of cyt *b* 559 by PS I and its reduction by PS II. Other groups have, however, reported the absence of this process in untreated tissue. The location of cyt *b* 559 in the electron transport chain connecting PS I and PS II is doubful. It has been suggested that cyt *b* 559 functions in a cycle or side path around PS II. It might be in an alternate pathway which functions when the normal electron donor to P 680 is not available.

(*vi*) *Cytochrome f (cyt),* a *c*-type cytochrome (MW 35,000) is also called cytochrome *c*554 or *c*552. It is an insoluble membrane bound protein with $E^{0'}$ = + 365 mV. Cytochrome *f* is in the reduced form in the dark and in the oxidised form in light. In photosynthetic bacteria cytochrome c_2, an analogue of cytochrome *f*, is present in several genera. Its $E^{0'}$ is between + 308 mV and + 350 mV.

(*vii*) *Plastocyanin (PC)* is a copper-containing protein which may be located immediately adjacent to P 700 of PSI. It is likely that plastocyanin may be the immediate electron donor to P700. It has a molecular weight of 10,000-20,000 with $E^{0'}$ = + 370 mV. Plastocyanin contains two Cu atoms which are the sites for oxidation reduction. It is blue in the oxidized state and colourless when reduced.

(*viii*) *Photosystem I (PSI) :* Kok (1956) showed that photosystem I contains a special form of chlorophyll *a* (called P 700) absorbing light maximally at around 700 nm. Excitation of P 700 by an absorbed photon causes an electron to be transferred from it to a primary acceptor. When P 700 loses an electron to the primary acceptor it leads to the formation of P^+ 700, a weak oxidant with $E^{0'}$ = 500 mV. This component is re-reduced by accepting an electron from PS II. The $E^{0'}$ of P 700 ws measured + 430 mV by Kok (1961). Recent determinations have given values of +520, + 493 and + 375 mV.

Photosystem I is associated with an important oxygen effect. Mehler (1971) first demonstrated that isolated chloroplasts generate hydrogen peroxide through a divalent phtoreduction of oxygen.

$$2e^- + O_2 + 2H^+ \xrightarrow[\text{mediator}]{\text{Autoxidisable}} H_2O_2$$

This reaction was demonstrated *in vivo* by Asada and Kiso (1973) who showed that isolated spinach chloroplasts generate H_2O_2 on illumination. H_2O_2 generation takes place in two steps.

In the first step there is univalent reduction of oxygen to form a superoxide radical.

$$O_2 + e^- \longrightarrow O_2^-$$

The enzyme *superoxide dismutase* (SOD) then converts the superoxide radical to H_2O_2.

$$O_2^- + O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Addition of cytochrome *c* to the reaction mixture stops production of H_2O_2 and cytochrome *c* is reduced. If SOD is added to the reaction mixture photoreduction of cytochrome *c* is inhibited. The entire sequence is as follows :

$$hu \rightarrow PSI \rightarrow PSI^{+} \xrightarrow{O_2} O_2 \rightarrow O_2 + SOD \longrightarrow H_2O_2$$

$$O_2 + Cyt\,a \rightarrow Cyt\,c + O_2$$

$$Fe^{+++} \quad Fe^{++}$$

Primary aceptor

(ix) Primary electron-acceptor of PSI : It was formerly thought that *NADP* was the primary electron of PSI. Subsequently it was suggested that *ferredoxin* was the primary acceptor. Still later Kok suggested that a compound which is a stronger reductant precedes ferredoxin. This substance is known by the general name of *ferredoxin reducing substance* (FRS).

The identity of the primary electron acceptor of PS I is controversial. The primary acceptor complex is perhaps closely associated with *ubiquinone* (UQ) and *bound ferredoxin,* and *iron-sulphur* (*Fe –S*) *protein.* The ESR spectrum indicates that the primary acceptor is the Fe-S protein. Stoichiometrically the abount of bound ferredoxin reduced is equal to the amount of P700 photooxidized. Redox titrations of PS I subchloroplast particles show that there are two Fd -type ESR signals. Both appear to be *4Fe-4S centres,* and are distinct from the signals of 2Fe-2S centres of *soluble ferredoxin. Centre A* is probably bound ferredoxin ($E^{0'} = -550$ mV]. *Centre B* has an $E^{0'}$ value of -590 mV. A 4 Fe-4S protein (MW 8,000), which may be bound ferredoxin, has been isolated and characterized to some extent.

McIntosh *et al.* (1975) have interpreted a new component (X^{-}) as the primary acceptor and consider bound ferredoxin to be a secondary acceptor. These observations have been later confirmed (Evans, Sihra and Cammack, 1976; McIntosh and Bolton, 1976; Ke *et al.* 1977). Sauer *et al.* (1978) suggest that there may be even *two* electron acceptors prior to bound ferredoxin.

Electrons are moved from the inner side of the membrane to the outer side, where they are accepted by the iron-sulphur (Fe-S) protein. Between the Fe-S protein and $NADP^{+}$ are two carriers, *ferredoxin* and *ferredoxin NADP* reductase.

(x) Ferredoxin (Fd) is a non-haeme iron-containing protein containing iron and sulphur (Fig. 17.1 J). Unlike in cytochromes the iron is not asscoiated with haeme. Ferredoxin has a relatively low molecular weight, 5,000 in bacteria and 13,000 in higher plants. Bacterial ferredoxin contains several iron atoms (7 in *Clostridium,* 6 in *Chromatium* and 5 in *Chlorobium*), while the ferredoxin of higher plants contains only two. The iron is alternately oxidized and reduced by accepting and donating electrons, respectively. The potential level of ferredoxin is –420 mV.

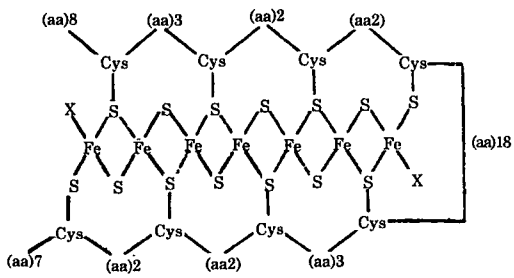


Fig. 17.1 (J). The prosthetic group of bacterial ferrodoxin (Fd), a nonhaeme iron protein.

(xi) Ferredoxin $NADP^{+}$ reductase is a soluble flavoprotein with FAD as the prosthetic group. The enzyme has a molecular weight of about 40,000. It contains one FAD, one essential sulphydryl group but no metal. Ferredoxin extracts two protons from the external medium to form $FADH_2$. $FADH_2$ finally donates the electron, which along with a proton, reduce $NADP^{+}$ to NADPH.

$$NADP + H^{+} \longrightarrow NADPH$$

(*xii*) *The ATP synthesizing enzyme complex* carries out photosynthetic phosphorylation, and drives the synthesis of ATP from ADP and Pi. The complex consists of the coupling factors CF_0 and CF_1 (knob). In contrast with the condition of the mitochondrial F_0-F_1 complex (ATPase), which is located on the inner (matrix) side of the crista, the chloroplast complex is situated on the outer side of the thylakoid membrane. This makes ATP directly available to the enzymes in the stroma space.

The coupling factor is composed of five subunits α, β, γ, δ, and ε. There is evidence from immunological studies indicating that the ATP binding site is located on the α-subunit and that the α and γ subunits are involved in photophosphorylation proper. When the coupling factor is treated with trypsin the γ, δ, and ε subunits are removed. The α and β proteins still remain active as ATPase but the coupling activity is lost. The ε- subunit acts as an inhibitor of ATPase.

(3) Electron Transfer Mechanism

(*i*) The overall reaction of photosynthesis is as follows :

$$6CO_2 + 6H_2O \xrightarrow[\text{Chlorophyll}]{\text{light}} C_6H_{12}O_6 + 6O_2$$

(*ii*) In the *light reaction* energy from the sun is captured in two molecules, adenosine triphosphate (ATP) and NADPH.

(*iii*) In the dark reaction glucose is synthesized from CO_2. The energy for the reaction is supplied by ATP and NADPH. Two molecules of NADPH and 3 molecules of ATP are required to reduce one molecule of CO_2.

$$6CO_2 + 12H_2O + 12NADPH + 18ATP \longrightarrow C_6H_{12}O_6 + 12\,NADP^+ + 6H^+ + 18\,ADP + 18Pi$$

(*iv*) The electron transfer chain of chloroplast thylakoids is involved in the generation of ATP and NADPH. The carrier molecules of the chain are similar to those of the respiratory chain of mitochondria, but the flow is in the opposite direction. The chain begins with water and ends with $NADP^+$. Electron flow is vectorial or unidirectional and follows a linear pathway.

(*v*) Electrons from water are utilized to reduce $NADP^+$ to NADPH. On accepting a pair of electrons and a proton $NADP^+$ is reduced to NADPH. The protons (hydrogen ions) and oxygen molecules are released from the membrane. The direction of proton translocation is opposite to that in mitochondria.

(*vi*) Electron transport from PS II to Q is from the inner to the outer surface of the membrane and takes place *against* the concentration gradient (from about + 800 mV to about zero mV). Each photon drives one electron across the membrane. Electron transfer from Q to P 700 of PS II is back across the membrane to the inner surface and takes place *along* the concentration gradient. Two electrons from P 680 along with two protons from the solution outside the membrane reduce PQ to PQH_2. The protons are released inside the membrane while the electrons continue along the chain. P 700 absorbs additional photons to again drive the electrons against gradient to the primary acceptor.

(*vii*) Electron flow is from inside the thylakoid membrane to the outside. The inside of the thylakoid sac therefore becomes positively charged and the outside negatively charged.

(*viii*) Protons accumulate in the thylakoid sac from two sources : (*a*) protons released on dissociation of H_2O into protons and electrons, and (*b*) protons pumped from outside to inside across the thylakoid membrane during electron transport.

(*ix*) Because the inside of the thylakoid sac has a much larger concentration of protons than the outside, an electrochemical gradient is created across the membrane. The proton gradient tends to drive protons from the inside to the outside of the membrane to equalize their concentrations. The proton gradient is a source of potential energy and drives the synthesis of

ATP from ADP and Pi in the ATPase complex (CF_1). Three protons apparently cross the complex for every molecule of ATP synthesized.

(4) ATP synthesis in photosynthesis

As mentioned previously, two molecules of NADPH and three of ATP are required to convert one molecule of CO_2 to a molecule of hexose such as glucose or fructose, *i.e.*, $1\frac{1}{2}$ molecules of ATP are needed per molecule of NADPH. One consistent finding regarding photophosphorylation was that one molecule of ATP is synthesized per two electrons passing down the electron transport chain. On this basis only one site of energy transduction was recognised. There is enough drop in potential in the electron transport chain connecting the upper end of PS II with the lower end of PS I to permit synthesis of ATP by electron transfer. It was believed that only one molecule of ATP is formed per pair of electrons passing down the chain.

Recently however several laboratories have reported ratios of ATP to two electrons exceeding 1, and in some cases approaching 2. *Thus there are two rather than one energy conservation sites. Site I* is generally considered to be located between plastoquinone and cytochrome *f* and *site II* between water and 'Q', the primary acceptor of PS II. Water or any other hydrogen carrying electron donor to PS II leaves behind a protron when oxidized by PS II. A proton gradient is thus created across the thylakoid membrane (*coupling site* II). The electron from water is accompanied by a proton on reduction of PQ. This proton has been removed from the outer side of the thylakoid vesicle and released into the thylakoid space when cytochrome *f* is reduced. This results in a further increase in the proton gradient (*coupling site* I).

The sequence of events in ATP formation is as follows :

(*i*) There is loose binding of ADP and phosphate to site I on the coupling factor.

(*ii*) The binding is tightened by energization, resulting in ATP formation at site I. There is simultaneous loosening and release of ATP at site II.

(*iii*) There is loose binding of ADP and phosphate to site II.

(*iv*) Tightening of binding by energization leads to ATP formation at site II. There is simultaneous loosening and release of ATP at site I.

Additional ATP is formed in chloroplasts by *cyclic photophosphorylation* though PSI. The high potential electron in reduced PS I acceptor or ferredoxin can be transferred through cytochromes b_6, cytochrome *f* and plastocyanin to oxidized P700 to generate ATP. Here ATP formation takes place without formation of NADPH and there is no oxygen generation. Cyclic photophosphorylation takes place when there is insufficient $NADP^+$ to accept electrons from reduced PS I acceptor or reduced ferredoxin.

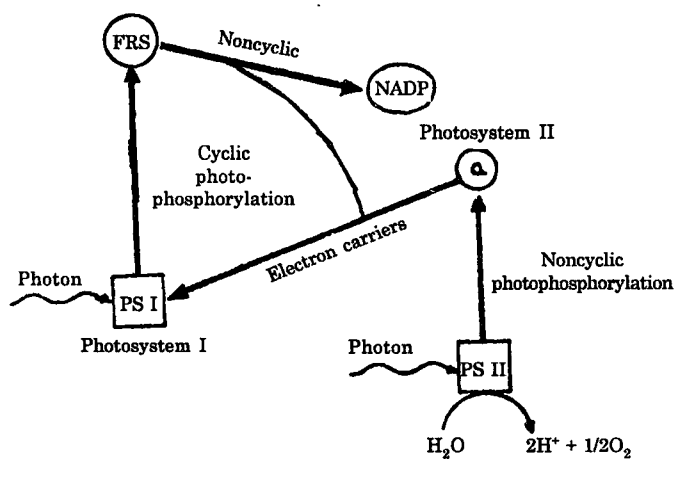


Fig. 17.1 (K). Outline of electron transfer in photosynthesis through cyclic and noncyclic photophosphorylation.

(5) Cyclic photophosphorylation

Cyclic phtophosphorylation (Fig.

17.1 L) takes place in the presence of light and chlorophyll. It is possible when the wavelength of light is more than 650mμ. The substrates required are ADP and P*i*, and the products are ATP and H_2O. The overall scheme of cyclic photophosporylation is as follows :

$$ADP + Pi \xrightarrow[\text{Chlorophyll}]{\text{Light}} ATP + H_2O$$

The details of cyclic photophosphorylation are as follows :

(*i*) The outer electron of chlorophyll absorbs light energy (*photon*). The electron is raised to an orbit more distant from the nucleus. In other words it is 'excited' or energized, and in this higher energy level is unstable.

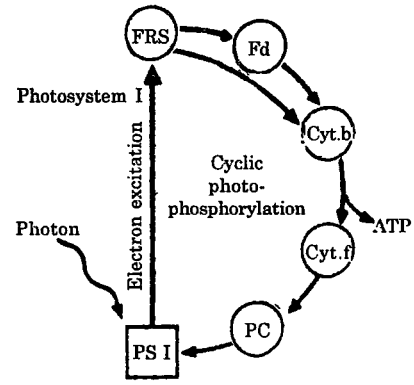


Fig. 17.1 (L). Cyclic photophosphorylation.

(*ii*) The electron is now accepted by an electron acceptor. *Ferredoxin-reducing substance* (*FRS*) (Fd-UQ) transfers electrons to ferredoxin. The chlorophyll which has lost the electron is now positively charged and can be designated as *CHl*$^+$

$$Chl \longrightarrow Chl^+ + e^-$$

(*iii*) The next electron acceptor is *cytochrome* b_6. The alternate pathways to cytochrome b_6 are from ferredoxin, or directly from FRS. A pathway from ferredoxin to plastoquinone has also been suggested, but is of a questionable nature.

(*iv*) The next two electron acceptors are *cytochrome f* and *plastocyanin* (PC). The redox potentials of both these compounds are very similar. Plastocyanin is the electron donor to P 700. (In some earlier accounts plastocyanin was placed before cytochrome *f* in the electron transport chain).

(*v*) As the electron passes from one component of the electron transport system to the next it progressively loses energy. In other words, there is a step by step lowering of the energy gradient. Some of the energy released is used to synthesize ATP from ADP and inorganic phosphate (P*i*). In other words, light energy is converted to chemical energy in the form of ATP.

$$ADP + Pi + Energy \longrightarrow ATP$$

(*vi*) The de-energized electron is finally returned to the chlorophyll molecule, which is now an electron acceptor. It will be seen that when the electron leaves chlorophyll is energy rich, but when it finally returns it is energy poor.

Cyclic photophosphorylation involves photosystem I only. In noncyclic photophosphorylation both the photosystems are involved. Cyclic photophosphorylation is so called because it functions in a closed loop. The chlorophyll molecule loses an electron to an acceptor and finally takes it back. Thus it is both an originator and an acceptor of electrons. In green plants both cyclic and noncyclic photophosphorylation apparently take place. The ATP formed in any one type of photophosphorylation is insufficient for the fixation of carbon dioxide during carbohydrate formation.

(6) Noncyclic photophosphorylation (Fig. 17.1 M)

In constrast to cyclic photophosphorylation there is evolution of oxygen in noncyclic photophosphorylation.

The flow of electrons is as follows :

$$Chl\ (PS\ II) \longrightarrow Q \longrightarrow PQ \longrightarrow Cyt.b_3(?) \longrightarrow (Cyt.f \longrightarrow PC \longrightarrow (PS\ I) \longrightarrow FRS(FD—UQ) \longrightarrow Fd \longrightarrow NADP^+$$

It will thus be seen that both pigment systems (photosystems I and II) are involved in the flow of electrons.

(1) In non-cyclic photophosphorylation the electrons are provided by water molecules. This is indicated by the fact that in tracer studies addition of water, whose oxygen has been labelled, gives rise to labelled oxygen gas. In pigment system II the quanta of light cause *'Photolysis' of water*. In the general sense this means that the water molecule is split into H^+ and OH^-.

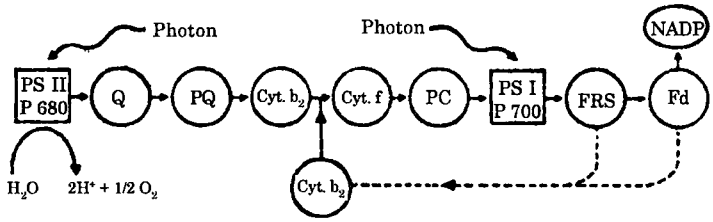


Fig. 17.1 M. Non-cyclic photophosphorylation.

Electron transport from pigment system II to compound Q takes place *against* the electrochemical gradient.

It is now possible to summarize the change in the potential gradient along the electron pathway. Electron flow normally takes place *along* the electrochemical gradient from the more electronegative components to the more electropositive ones. Thus in Fig. 17.1(I) normal electron flow would be from above downwards. *i.e.,* from – 600 mV to 800 mV. The two light reactions in pigment systems I and II, however, provide the energy for electron *flow against* the electrochemical potential gradient. Pigment system II changes the energy level from + 800 mV to about – 130 mV and pigment system I from about + 500 mV to about –600 mV. Thus from pigment system I to the FRS, and from pigment system II to compound Q, the transport of electrons is *against* the potential gradient. Between compound Q and P 700 of pigment system I, electron transport takes place *along* the potential gradient so also electron transport from the FRS to $NADP^+$.

Table 17.1

Cyclic photophosphorylation	*Non-cyclic photophosphorylation*
1	*2*
1. Only photosystem I functions in cyclic photophosphorylation.	1. Both photosystems I and II function in non-cyclic photophosphorylation.
2. Cyclic photophosphorylation functions in a closed loop. Electrons released from chlorophyll to acceptor return to chlorophyll.	2. An independent electron donor is necessary. Water is the ultimate source of electrons. $NADP^+$ is the final electron acceptor.
3. There is no net production of reduced compounds. $NADPH_2$ is not formed and assimilation of CO_2 is retarded.	3. $NADPH_2$ is the reduced compound formed. It is utilized in carbon assimilation.
4. Oxygen is not evolved.	4. ATP formation is coupled to evolution of oxygen.

1	*2*
5. In bacteria only cyclic photophosphorylation takes place.	5. In green plants noncyclic photophosphorylation also occurs.
6. Cyclic photophosphorylation is not sensitive to either antimycin or dichlorophenyl dimethyl-urea (DCMU).	6. DCMU inhibits flow of electrons from water to $NADP^+$ and thus stops noncyclic photophosphorylation.
7. The flow of electrons is as follows : CHl(PS-I)—FRS—FD—Cyt.b_6—Cyt.f— PC—Chl (PS–I)	7. The flow of electrons is as follows : PS—II—Q—PQ—Cyt.f—PC—PS-I—FRS—FD—$NADP^+$

(C) The Dark or Blackman Reaction : Calvin Benson Cycle

During the dark reactions of photosynthesis carbon dioxide is reduced to form carbohydrates. The term 'dark reaction' implies that the reaction is not dependent on light. Synthesis of carbohydrates proceeds in the dark. The dark reaction has been worked out mainly by Calvin, Benson and Bassham. The pathways by which carbon divide is fixed into carbohydrates is called the *Calvin Benson cycle* or *Calvin Bassham cycle.* Carbon dioxide and water are used to generate carbohydrate in the presence of ATP and NADPH.

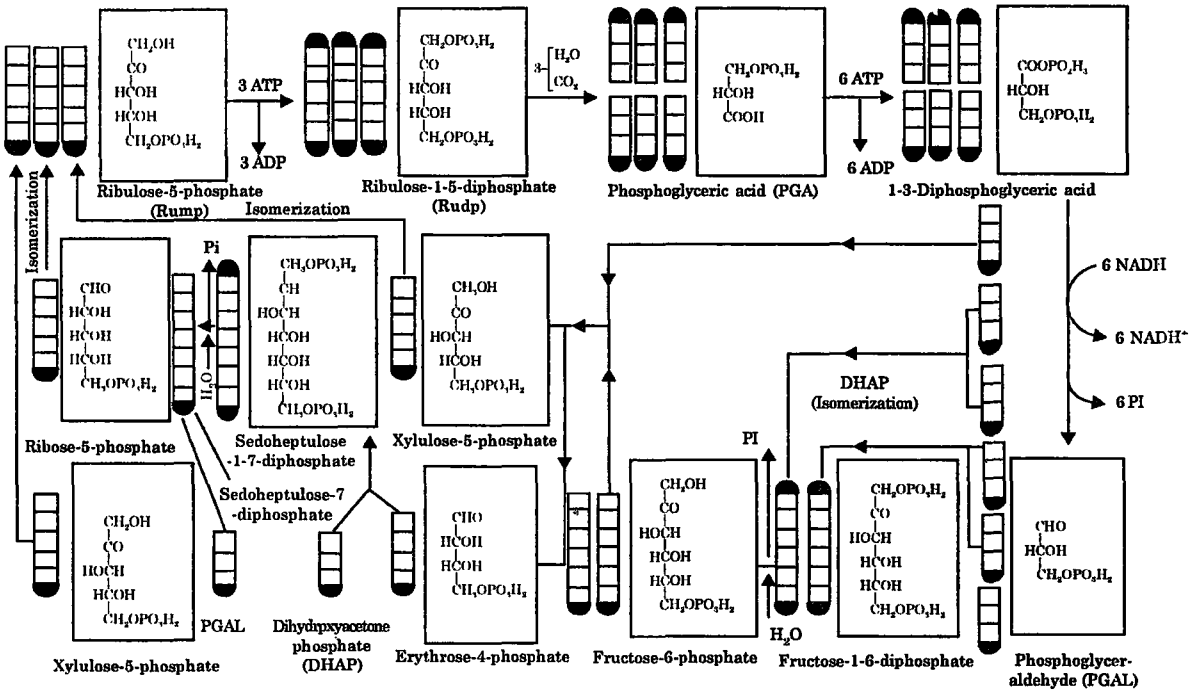


Fig. 17.1 (N). The dark reactions : Calvin Benson cycle. Only 3 of the 6 ribulose-5 phosphates are shown, and the cycle is halved accordingly.

Outline of the dark reactions

(1) 6 Ribulose-1,5-diphosphate + $6CO_2$ + $6H_2O$ ⟶ 12 3-Phosphoglyceric acid
(RudP) (3 PGA)

(2) 12 3-Phosphoglyceric acid (3 PGA) + 12 ATP ⟶ 12 1,3-Diphosphoglyceric acid + 12 ADP

1,3-Diphosphoglyceric acid + 12 NADPH ⟶ 12 Phosphoglyceraldehyde (PGAL) + 12 NADP + 12 Pi

(3) 5-Phosphoglyceraldehyde (PGAL) ⟶ 5-Dihydroxyacetone phosphate (DHAP)

3 PGAL + 3 Dihydroxyacetone phosphate (DHAP) ⟶ 3 Fructose 1-6 diphosphate

3 Fructose 1-6 diphosphate + 3 H_2O ⟶ 3 Fructose-6-phosphate + 3 Pi

(4) 2 Fructose-6-phosphate + 2 PGAL ⟶ 2 Xylulose-5-phosphate + 2 Erythose 4-phosphate

(5) 2 Erythrose-4-phosphate + 2 DHAP ⟶ 2 Sedodheptulose-1-7-diphosphate

2 Sedoheptulose-1, 7-diphosphate + $2H_2O$ ⟶ 2 Sedoheptulose-7 phosphate + $2P_i$

(6) 2 Sedoheptulose-7-phosphate + 2 PGAL ⟶ 2 Ribose-5-phosphate + 2 Xylulose-5-Phosphate

2 Ribose-5-phosphate ⟶ 2 Ribulose-5-phosphate

4 Xylulose-5-phosphate ⟶ 4 Ribulose-5-phosphate (Ribose monophosphate) (Rump)

6 Ribulose-5-phosphate + 6 ATP ⟶ 6 Ribulose 1-5 diphosphate + 9 ADP

Sum : $6CO_2$ + 18 ATP + 12 NADPH + $12H^+$ + 11 H_2O ⟶ Fructose-6-phosphate + 18 ADP + 12 $NADP^+$ + $17H_3PO_4$ (Pi)

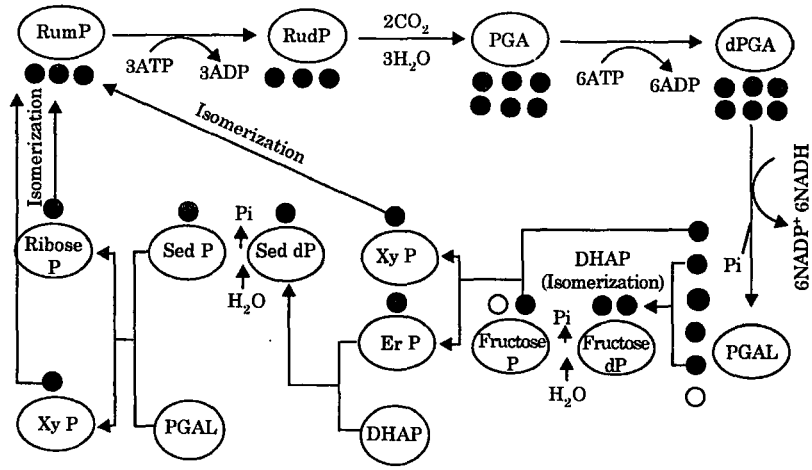


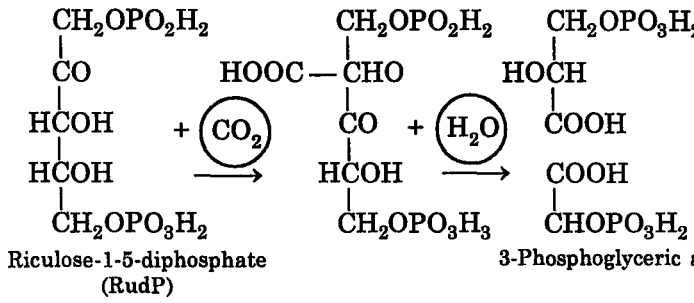
Fig. 17.1 (O). Outline of the dark reaction.

(1) Production of PGA

Calvin and his co-workers found that the first product to accumulate during photosynthesis was *phosphoglyceric acid* (PGA). This arises as follows.

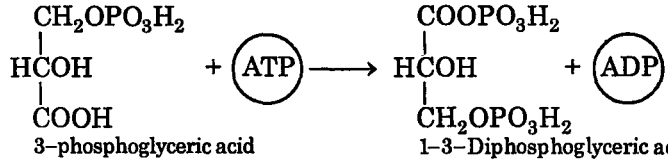
Carbon dioxide is first attached to *ribulose-1-5-diphosphate* (*Rud* P) a 5-carbon atom compound to form an *intermediate 6-carbon compound*. Each molecule of this compound then splits to form two molecules of *PGA*. Radioactive carbon dioxide ($^{14}CO_2$) was used in the experiment, and this CO_2 contributed one carbon atom. Of the two PGA molecules formed only one has radioactive carbon. Thus only one free molecule of PGA is formed per molecule of CO_2 entering the cyclic.

Actually, 6 molecules of RudP and 6 molecules of CO_2 react to produce 12 molecules of PGA.

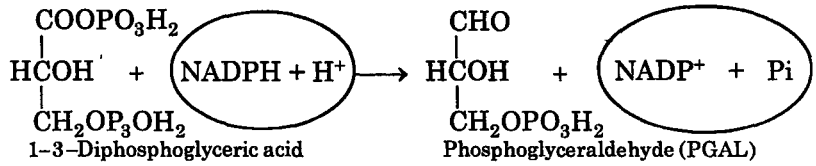


(2) Production of PGAL

Phosphoglyceric acid (PGA) is reduced to *phosphoglyceraldehyde* (PGAL). This process is the reverse of the oxidation step in glycolysis when PGA is oxidized to PGAL. In all 12 molecules of PGAL are produced from the 6 molecules of Rud P. The reaction takes place in two steps. Firstly, PGA is phosphorylated by ATP to 1, 3-*diphosphoglyceric acid.*

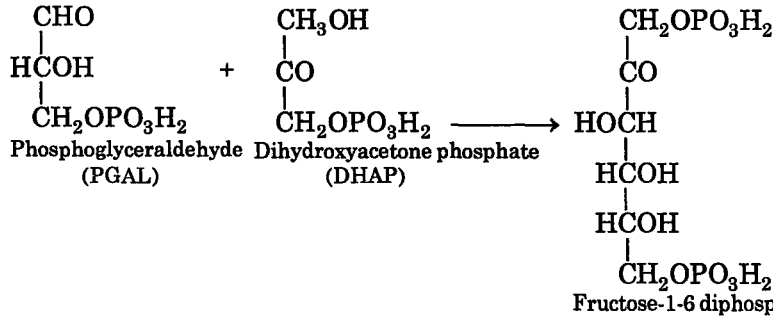


Secondly, 1, 3-*diphosphoglyceric acid* is reduced by NADPH + H^+ to *phosphoglyceraldehyde* (*PGAL*)



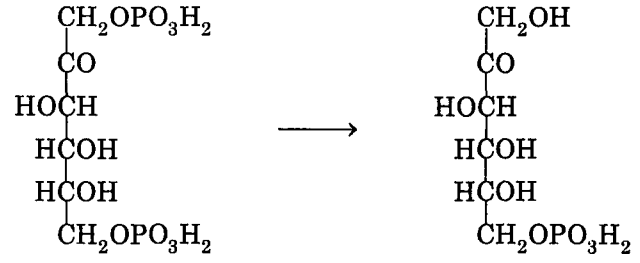
(3) Production of Fructose-6-phosphate

PGAL is converted into its isomer *dihydroxyacetone phosphate* (DHAP), as in glycolysis. DHAP condenses with PGAL to form *fructose-1-6-diphosphate.*



This process is the reverse of the breakdown of fructose 1-6-diphosphate in glycolysis.

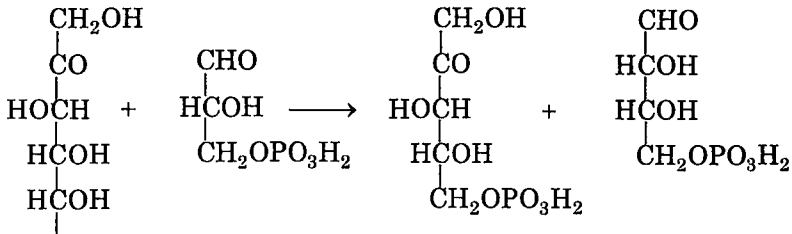
One phosphate group is removed from *fructose 1-6-diphosphate* (*dephosphorylation*) resulting in the formation of *fructose-6-phosphate.*



Fructose 1-6-diphosphate Fructose-6-phosphate

(4) Production of Xylose phosphate

Fructose-6-phosphate reacts with PGAL to yield a pentose (5C), *xylulose-5-phosphate,* and tetrose (4C), *erythrose-4-phosphate.* The reaction is catalysed by the enzyme *transketolase.*

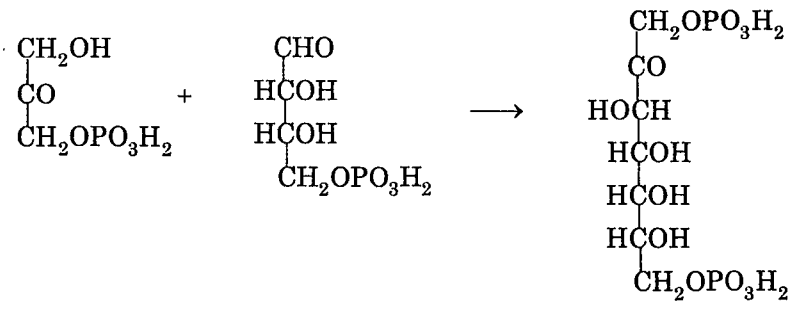


Fructose 1-6-phosphate Xylulose-5-phosphate Erythorse-4-phosphate

Xylulose-5-phosphate readily isomerizes to *ribulose-5-phosphate.*

(5) Formation of Sedoheptulose-1, 7-diphosphate

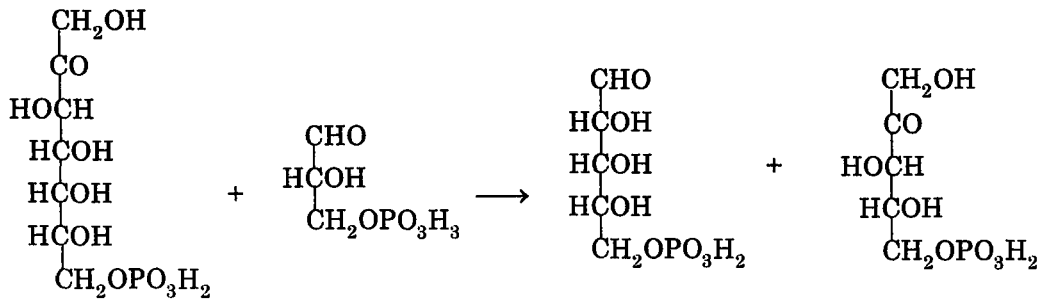
Erythrose-4-phosphate reacts with DHAP to form *sedoheptulose 1-7-diphosphate,* the reaction being catalysed by the enzyme *transladolase.*



Erythrose-4-phosphate DHAP Sedoheptulose-1-7-diphosphate

(6) Formation of Ribose-5-phosphate

Sedheptulose-7-phosphate reacts with another molecule of PGAL to form *ribose-5-phosphate* and *xylulose-5-phosphate.* Both these products readily isomerize to *ribulose-5-phosphate* or *ribulose monophosphate* (RumP).



Sedoheptulose-7-phosphate + PGAL ⟶ Ribose-5-phosphate + Xylulose-5-phosphate

Special Adaptations

The reductive pentose pathway (Calvin-Benson cycle) is utilized for CO_2 assimilation in all land plants investigated. The preliminary carboxylation system between atmospheric CO_2 and the ribulose diphosphate carboxylation reaction is of two types (A) Crassulacean, acid metabolism (CAM) and (B) the C_4 pathway of Hatch-slack Kortschak (HSK) pathway (See Table below).

Table 17.2. Families having C_4 and CAM mechanisms of photosynthesis

C_4 Mechanism	*CAM Mechanism*
Monocotyledons	
Cyperaceae	Agavaceae
Gramineae	Bromeliaceae
	Liliaceae
	Orchidaceae
Dicotyledons	
Aizoaceae	Aizoaceaa
Amaranthaceae	Asclepiadaeae
Chenopodiaceae	Bataceae
Compositae	Cactaceae
Euphorbiaceae	Caryophyllaceae
Nyctaginaceae	Chenopodiaceae
Protulacaceae	Compositae
Zygophyllaceae	Convolvulaceae
	Crassulaceae
	Euphorbiaceae
	Plantaginaceae
	Portulacaceae
	Vitaceae

(A) Crassulacean acid metabolism (CAM) (Fig. 17.1 P)

The members of the family Crassulaceae have a special type of metabolism called crassulacean acid metabolism (CAM) which is also exhibited by members of other families. CAM plants are succulents with thick fleshy leaves, or, when leaves are absent, a swollen photosynthetic stem. CAM plants can synthesize large amounts of *malic* and *isocritic acids* at night. Photosynthesis occurs during the day and these acids disappear. The stomata of the leaves remain closed during

the day and open only at night. This is an adaptation to conserve water, since succulents exhibiting CAM are found in dry habitats. During the night CO_2 is taken into the leaves through the open stomata. Because photosynthesis is limited by the storage pool of organic acid and carbohydrate, CAM plants are generally slow growing.

The CAM mechanism shows various modifications. Well watered *Agave americana* shows some normal daytime photosynthesis along with some CO_2 fixation at night. In watered *Agave deserti,* however, dark carboxylation stops and is replaced by normal C_3 daytime photosynthesis.

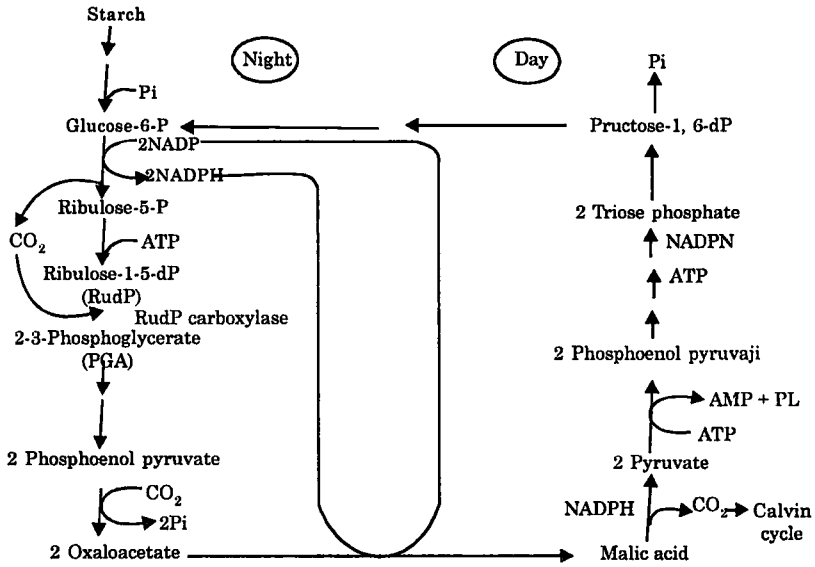


Fig. 17.1 (P). Crassulacean acid metabolism.

Reactions of CAM

(1) Formation of oxaloacetate : The requirements for formation of oxaloacetate are CO_2 and *phosphoenol pyruvic acid* (PEP). PEP is formed from stored carbohydrate in leaves, particularly starch. In the CAM species the stomata remain open at night and CO_2 entering the leaves is fixed by PEP to form *oxaloacetate.*

$$CO_2 + PEP \longrightarrow \text{Oxaloacetate} + Pi$$

(2) Formation of malic acid : Oxaloacetate is reduced by *malic dehydrogenase to malic acid,* which accumulates in the vacuoles of leaf cells. During this step $NADPH_2$ formed in the pentose phosphate pathway is utilized, and the NADP formed enters the pentose phosphate pathway.

$$\text{Oxaloacetate} \xrightarrow{NADPH_2 \longrightarrow NADP} \text{Malic acid}$$

(3) Release of CO_2 from malic acid : During the day ATP and NADPH are abundantly available from the photosynthesis reactions. The stomata of the leaves are closed and malate is transported back out of the vacuoles to the cytoplasm. Here malic acid is decarboxylated by an NADP linked *malic enzyme* to yield pyruvate and CO_2. Thus the CO_2 stored at night is made available for synthesis. The relase of CO_2 from malic acid is by the same mechanism as employed by C_4 plants.

$$\text{Malic acid} \xrightarrow[\text{malic enzyme}]{NADP \longrightarrow NADPH_2} \text{Pyruvate} + CO_2$$

(4) Formation of sugars : Photoactivation of the reductive pentose pathway in chloroplasts results in the reduction of CO_2 released from malic acid to hexose sugar through this pathway.

Malic acid ⟶ CO_2 ⟶ RudP ⇅ PGA ⟶ Hexose (Reductive pentose phosphate cycle)

The Hatch-Slack-Kortschak Pathway (Fig. 17.1 Q)

In the Calvin-Benson cycle the first stable compound formed is *phosphoglyceric acid* (PGA), which has three carbon atoms. Plants in which PGA is the first-formed substance are therefore called C_3 *plants*. The Russian scientists I.A. Tarcheveski and Y.S. Karpilov (1963), however, questioned the universal operation of the Calvin Benson cycle in plants. In 1963 Kortschask, Hart and Burr reported that malate and aspartate were the major products formed in sugarcane leaves. This observation was conformed by Hatch and Slack (1966). They showed that during short periods of photosynthesis in sugarcane leaves, four–carbon compounds like *oxaloacetate malate* and *aspartate* were formed. The pathway in which these compounds are formed has been called the *hatch-Slack Kortschak (HSK) pathway*. Plants in which the cycle takes place are called c_4 *plants*.

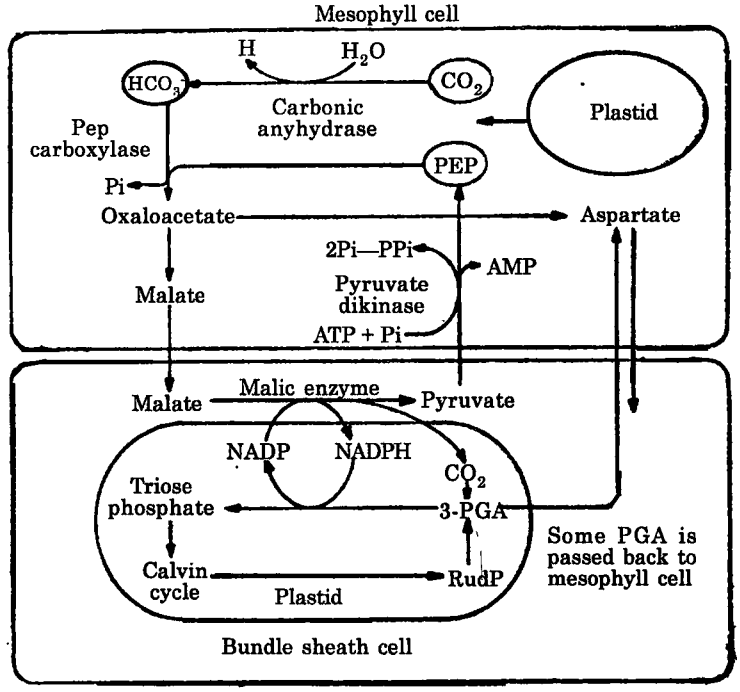


Fig. 17.1 (Q). Diagram of the HSK pathway (C_4 cycle)

The C_3 plants have been called "*non-efficient plants*" by some authors because they cannot grow fast at high temperatures and light intensities. They carry out carbon fixation through the Calvin-Benson 3-carbon pathway. The C_4 plants are called "*efficient plants*", because they grow fast at high temperatures and light intensities. The predominant pathway for carbon fixation in C_4 plants is the *HSK pathway,* although the *Calvin-Benson cycle* also functions along side. C_4 plants are mostly *tropical* plants growing in regions of high light intensities and temperature. They include *monocots* like maize, rice, wheat, corn, sorghum and sugarcane. Some *dicots* also, *e.g.,* members of the families Amaranthaceae and Chenopodiaceae have the C_4 dicarboxylic acid pathway of photosynthesis and carbon dioxide fixation (Laetsch, 1968).

As far as *utilization of carbon dioxides* is concerned, C_4 Plants utilizing the HSK pathway are more efficient than C_3 plants which utilize the Calvin-Benson cycle. In C_3 plants up to 20% of the carbon dioxide initially fixed into carbohydrates is released as carbon dioxide. In C_4 plants, also, photorespiration results in the breakdown of carbohydrates to carbon dioxide and water. The carbon dioxide is however, trapped and incorporated into the C_4 *oxaloacetate*. Therefore, all the carbon dioxide taken up is utilized for plants growth.

C_4 plant leaves have what is called 'Kranz anatomy.' A single layer of bundle sheath cells around the vascular bundle is surrounded by a loosely packed layer of mesophyll cells. In tropical grasses the chloroplasts of *bundle sheath cells* differ from those in *mesophyll cells*. That monocots have two types of chloroplasts was pointed out as early as in 1904 by the German botanist G. Habertandt. The light microscopy studies of Rhoades and Carvalho (1944) demonstrated chloroplast dimorphism in maize and sorghum. Later electron microscope studies on maize (Hodge, McLean and Mercer, 1955), many tropical grasses (Johnson, 1964) and sugarcane (Laetsch, Stetler and Vlitos, 1965) have confirmed this dimorphism.

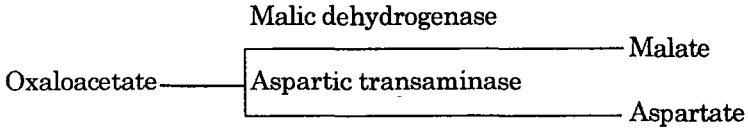
The *bundle sheath cells* around vascular tissue have large chloroplasts that are without grana and have many starch grains. The *mesophyll cells* contain smaller chloroplasts which possess well developed grana and have few starch grains. It has been found that bundle sheath cells apparently use the 3-*carbon* (*Calvin-Benson*) pathway, while the mesophyll cells predominantly use the 4 *carbon* (*HSK*) pathway.

Reactions of the HSK Pathway

(i) Formation of oxaloacetate : This reaction takes place in the cytosol of mesophyll cells. CO_2 is converted into a bicarbonate ion (HCO_3^-) by *carbonic anyhydrase*. The 3C compound *phosphoenol pyruvate* (PEP) is carboxylated to form oxaloacetate, a 4C compound. The reaction is catalysed by *PEP carboxylase*, which is present in large amounts in mesophyll cells.

$$PEP + CO_2 + H_2O \xrightarrow{\text{PEP carboxylase}} \text{Oxaloacetate} + H_3PO_4$$

(ii) Formation of malate and aspartate : Oxaloacetate is very unstable and is converted to either *malate* or *asparate*. Oxaloacetate is reduced to *malate* by light-generated $NADPH_2$, the reaction being catalysed by *malic dehydrogenase*. Oxaloacetate is converted to *asparate* by an *aspartic transminase*.



These reactions also take place in the cytosol of mesophyll cells. Malate and aspartate are then transported to bundle sheath cells.

(iii) Formation of pyruvate : In the bundle sheath cells malate undergoes oxidative decarboxylation to *pyruvate*. The reaction is catalised by *malic enzyme*.

$$\text{Malate} + NADP^+ \xrightarrow{\text{Malic enzyme}} \text{Pyruvate} + NADPH + H^+ + CO_2$$

In some C_4 species the *aspartate* undergoes *transmination to oxaloacetate* which is then presumably *decarboxylated* to *pyruate*. The transamination is catalysed by *asparate transminase*.

$$\text{L-Aspartate} \xrightarrow{\text{Transamination}} \text{Oxaloacetate} \xrightarrow{\text{Decarboxylation}} \text{Pyruvate}$$

(iv) Formation of PEP : The pyruvic acid produced in reaction 3 is transported back to the mesophyll cells. Here the enzyme pyruvate *phosphate dikinase* phosphorylates pyruvic acid to

phosphoenol pyruvate (PEP). This enzyme is unusual in that it splits ATP into AMP and PPi. PPi is then degraded to Pi.

$$\text{Pyruvate} + \text{ATP} + H_3PO_4 \xrightarrow[\text{dikinase}]{\text{Pyruvate}} \text{PP} + \text{AMP} + \text{PPi}$$

(*v*) The CO_2 and NADPH generated in the chloroplasts of bundle sheath cells are utilized in the Calvin-Benson cycle or carboxylation of *ribulose diphosphate* (RudP) to synthesize 3-*phosphoglycerate* (PGA). There is now evidence that 85% of the CO_2 used in bundle sheath cells comes from the C_4 cycle and only 15% is diffused atmospheric CO_2.

$CO_2 \longrightarrow$ 3PGA $\longrightarrow$ Triose phosphate
$\uparrow$ $NADPH_2 \longrightarrow$ NADP $\downarrow$
RudP $\longleftarrow$ Calvin cycle

(*c*) Photorespiration and Glycollic acid Metabolism (Fig. 17.1 R)

Although C_3 plants respire in the dark, the rate of oxygen utilization increases markedly when the plants are illuminated. Photorespiration is a light-driven efflux of CO_2 which proceeds alongside with net CO_2 influx during photosynthesis. Photorespiration may attain 50% of the net rate of photosynthesis. Photorespiration results in CO_2 evolution in light. This has the net effect of decreasing photosynthesis which takes up CO_2 in light. It is therefore a wasteful process which prevents plants from achieving a maximum yield in photosynthesis. In crop species the yield would be greater if photorespiration did not occur. The substrate for photorespiration is *glycollate*. Breeding of plants with lower photorespiration rates, or inhibiting glycollate synthesis, would be means of increasing crop yields.

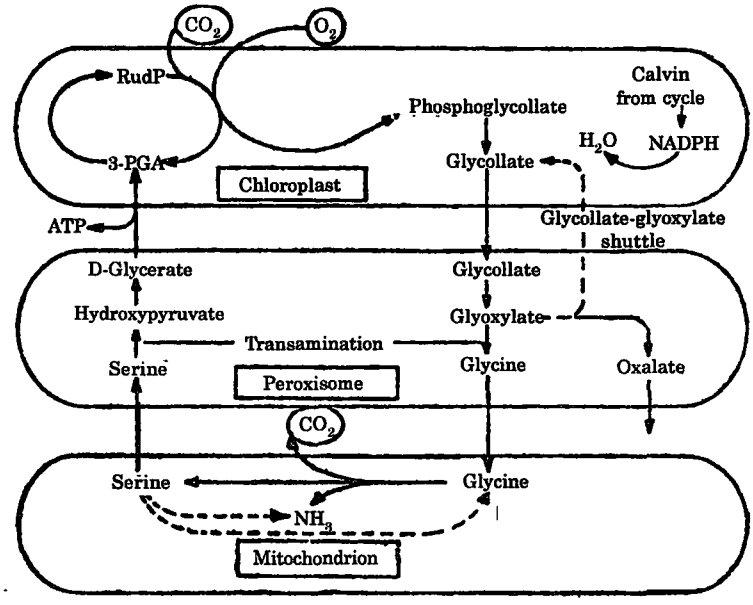


Fig. 17.1 (R). Some pathways in glycollate metabolism.

Photorespiration is exhibited by crop plants like wheat, rice, other cereals, many legumes and sugar beets, while crops like corn, sorghum and sugar cane do not have photorespiration.

The *CO_2 compensation point* is the CO_2 concentration at a given constant light intensity at which there in a balance between photosynthetic assimilation and respiration. The enzymes of the photorespiratory pathway are present in both C_3 and C_4 plants. The CO_2 compensation point for common C_3 crop plants is about 40-60 ppm at 25°C, while that for C_4 plants is often less than 10 ppm. The CO_2 generated in C_4 plants during photorespiration is trapped and recycled internally by cytoplasmic *PEP carboxylase* of mesophyll cells. Thus CO_2 efflux is prevented.

Glycollic acid is a 2-carbon compound which is formed in large quantities in the chloroplasts of C_3 plants, from where it moves out into the cytosol.

Reaction of Glycollate Metabolism

(i) In chloroplasts : Phosphoglycollate is probably one of the sources of *glycollate.* The formation of phosphoglycollate is catalysed by *ribulose disphosphate (RudP) carboxylase* which is found in chloroplasts. Other sources of glycollate are organic acids such as *acetate* and a *transketolase* which may yield *glycoaldehyde* as a side product. Glycoaldehyde is then oxidised to *glycollate.*

(ii) In peroxisomes : Glycollate is rapidly metabolized in the peroxisomes (microbodies). A *flavoprotein oxidase* converts glycollate into *glyoxylate* with the formation of H_2O_2. Most of the H_2O_2 is probably destroyed by *peroxidases* or *catalase.* Some of it may react non-enzymatically with glyoxyllate, decarboxylating it to *formate* and CO_2. Glyoxylate undergoes *transamination* to *glycine.*

(iii) In mitochondria : Glycine can be decarboxylated in the mitochondria. It can also be converted to *serine.*

(iv) In peroxisomes : Some of the serine may go to the peroxisomes where it is oxidized to *hydroxypyruvate and glycerate.*

(v) In chloroplasts : In the chloroplasts glyceric acid can be synthesized into glucose.

Biosynthesis of Disaccharides (Sucrose)

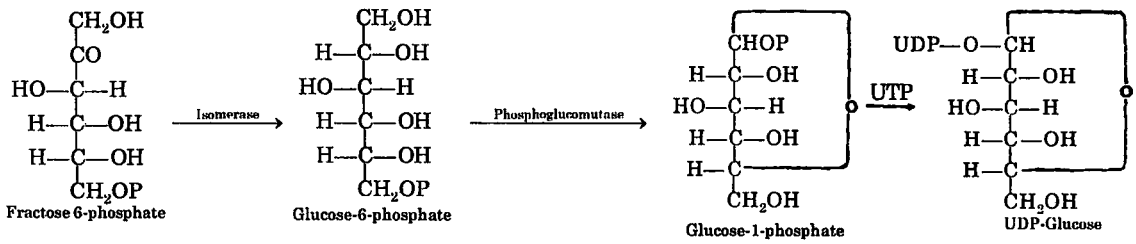
Fructose 6-phosphate formed in the dark photosynthesis is converted into glucose 6-phosphate by isomerase, and glucose 6-phosphate into glucose 1-phosphate by phosphoglucomutase.

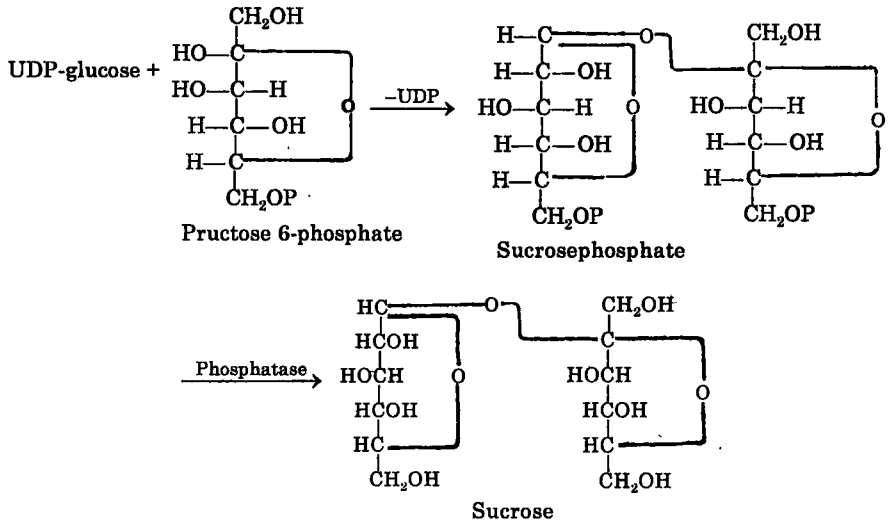
Glucose 1-phosphate now reacts with UTP (uridine triphosphate) to form UDP glucose, the glucose molecule of which exchanges with the proton of the 2-hydroxyl group of fructose 1-phosphate or fructose 6-phosphate to form sucrose and UDP. In plants, sucrose is synthesised by the following reactions :

(*i*) UDP-glucose + Fructose $\rightleftharpoons$ Sucrose + UDP

(*ii*) UDP Glucose + Fructose 6-phosphate $\longrightarrow$ UDP + sucrose phosphate $\xrightarrow{\text{Phosphatase}}$ Sucrose.

In terms of structural formulate, the biosynthesis of sucrose may be summarised as follows :



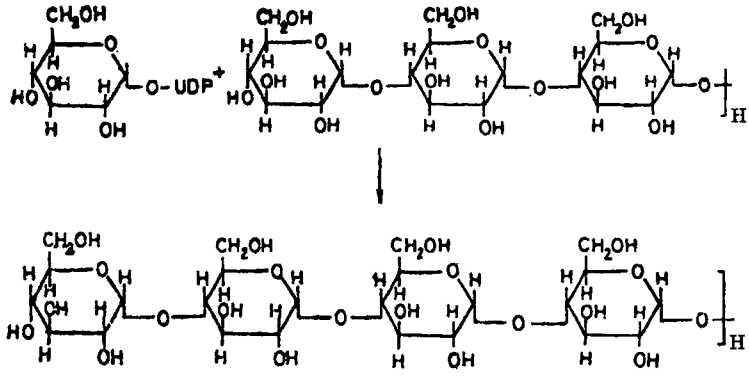


Biosynthesis of Polysaccharides

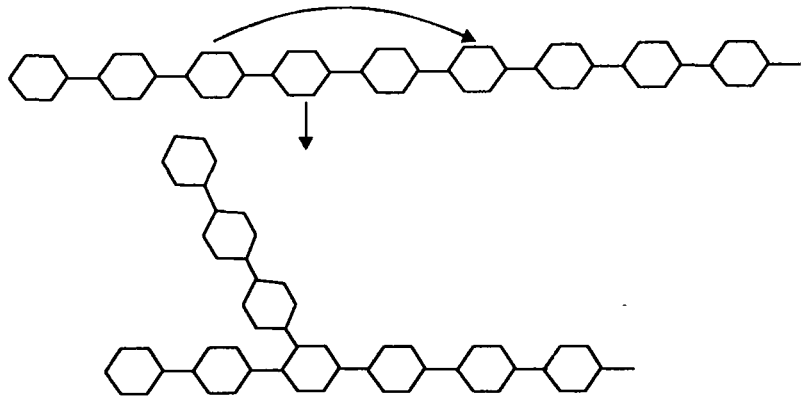
Polysaccharides are produced by monosaccharide phosphate by the action of enzymes. The biosynthesis of starch *in vitro* has been extensively studied in the presence of enzymes. Leloir's discovery of uridine diphosphate glucose as a precursor in the biosynthesis of starch was immediately followed by the discovery of other nucleoside diphosphate derivatives of sugars. These precursors, together with specific enzymes, are responsible for the formation of glycons in general. In the cells, nucleoside diphosphate derivatives of sugars are formed as part of the activation process in accordance to the following reaction :

Nucleoside triphosphate + Sugar-1-Phosphate $\rightleftharpoons$ Nucleoside diphosphate sugar + Pi

For glycogen synthesis, the enzyme used is glycogen synthetase. This enzyme catalyses the formation of linear polymers of glucose which are connected through α-1, 4 glycosidic bonds. The reaction is started on a *primer* which is a trace of readymade glycogen, a branched polymer. In this case, the role of the enzyme is to add to the non-reducing end of branches of glucose molecules from UDP glucose.



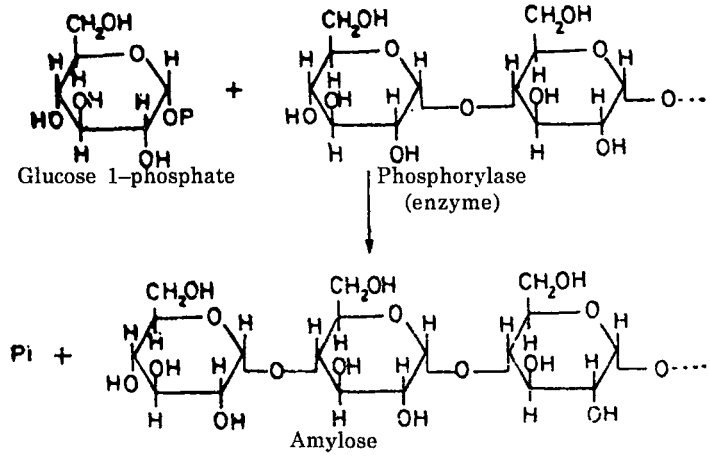
When the chains grow to become 10 units or more glucose units long, these are attacked by a second enzyme called transglycosydase. The role of the latter enzyme is to catalyse cleavage of several glucose units and their immediate condensation on the same branch or another branch. As transglycosydase is specific for α-1, 6 glycosidic bonds, branches are formed as depicted schematically here :



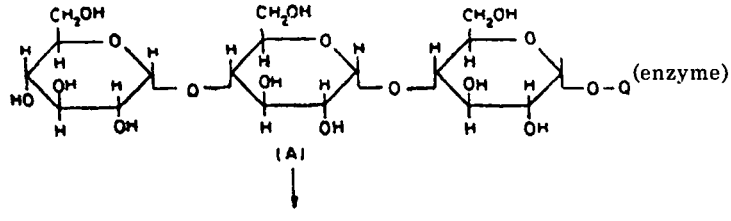
In the biosynthesis of starch, UDP-glucose may act as a precursor. However, other nucleoside diphosphate derivatives have also been found to be more effective. It is likely that ADP glucose is the precursor of starch.

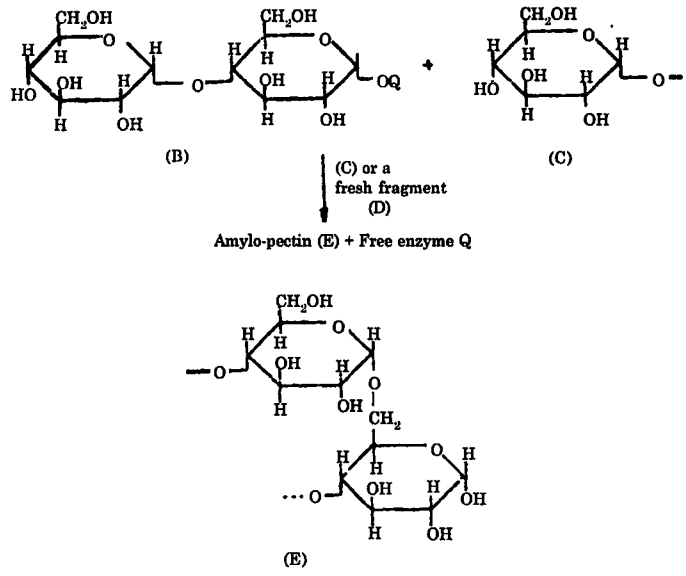
In some microorganisms, cellulose is produced from UDP-glucose while in plants, it is produced from GDP glucose. The same mechanism is also applicable to other homoglycans.

Amylose : The biosynthesis of amylose in which glucose units are linked by 1, 4′ linkages may be represented a follows :



Amylopectin : In amylopectin molecule, some of the glucose units are linked through 1, 4′ linkages while some other through 1, 6′ linkages. Thus, the synthesis of amylopectin from glucose 1 phosphate involves two step :





(*i*) In the first step, amylose units are synthesised as in amylose.

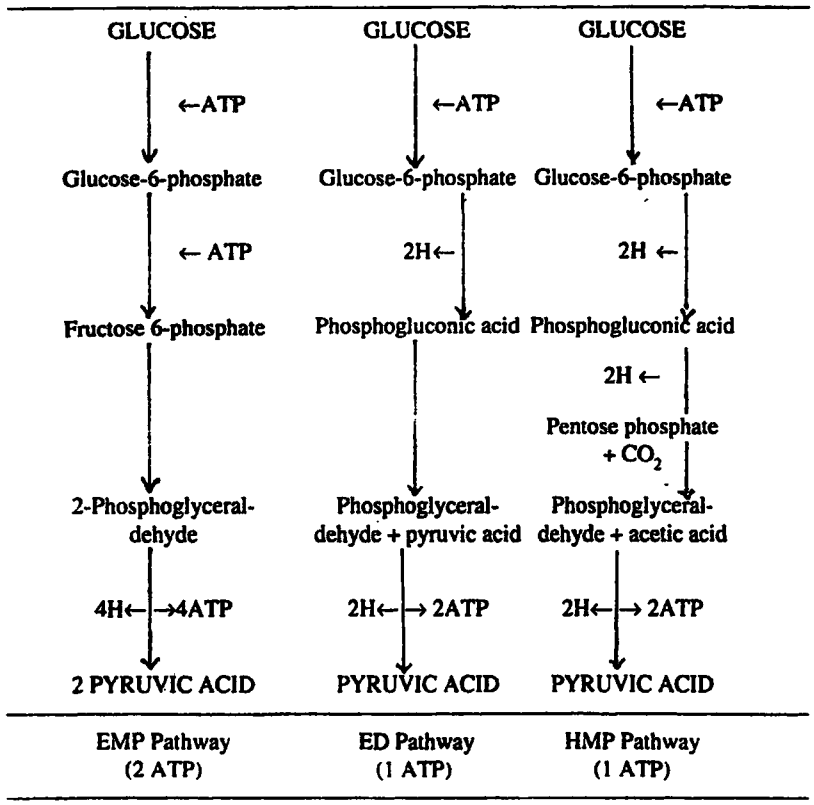


Fig. 17.2. Some of the pathways of glycolysis.

(*ii*) In the second step, Q-enzyme attackes amylose units to form amylopectin. The role of Q enzyme is to break amylose-substrate (A) into a shorter chain amylose enzyme complex (B) and a short amylose fragment (C). The complex (B) further reacts with another fragment of amylose either C or fresh fragment so as to form 1, 6′-branched products amylo-pectin (E) and the free enzyme Q.

Biological Oxidation of Carbohydrates

There are four major pathways of carbohydrate breakdown in microorganisms. These pathways result in the breakdown of sugars to the key metabolite *pyruvate*. The pathway are :

I. Glycolysis : The Embden-Meyerhof Parnas (EMP) pathway.
II. The hexose monophosphate (HMP) pathway (pentose phosphate pathway or Warburg-Dickens pathway).
III. The Entner-Duodoroff (ED) pathway.
IV. The phosphoketolase pathway (Fig. 17.2).

Table 17.1

CARBOHYDRATE METABOLISM

(A) Pathways Leading to Pyruvate Formation
I. Glycolysis : Embden-Meyerhof Parnas (EMP) scheme.
II. Hexose monophosphate (HMP) pathway (pentose phosphate or Warburg-Dickens pathway).
III. Enter-Duodoroff (ED) pathway.
IV. Phosphoketolase pathways.
  1. Pentose phosphoketolase pathway.
  2. Hexose phosphoketolase pathway.

(B) Aerobic Pathways of Pyruvate Metabolism
I. Krebs tricarboxylic acid (TCA) cycle or citric acid cycle.
II. Electron transport.
III. Glyoxylate cycle (special modification of the TCA cycle).

(C) Fermentation
I. Alcoholic fermentation
II. Lactic acid fermentation
  1. Homolactic fermentation
  2. Heterolactic fermentation
III. Propionic acid fermentation
  1. Propionic acid fermentation from glucose
  2. Propionic acid fermentation from lactate
IV. Formic acid fermentation
  1. Mixed acid producers
  2. Butanediol producers
  3. Butyric acid producers

(A) Pathways Leading to Pyruvate Formation

I. Glycolysis (Embden-Meyerhof-Parnas pathway)

(Fig. 17.3, 17.4 and 17.5)

The sequence of reactions which convert glucose to pyruvic acid along with the production of ATP is known as *glycolysis*. The most common pathway in glycolysis is the *Embden-Meyerhof-*

Parnas (*EMP pathway*. It is found in many organisms, both prokaryotes and eukaryotes. In aerobic organisms glycolysis leads to the *Kerbs citric acid cycle* and the electron transport chain, which release most of the energy contained in glucose. Under aerobic conditions pyruvate enters the *mitochondria* where it is completely oxidized to CO_2 and H_2O. In actively contracting muscle, where there is insufficient oxygen supply, pyruvate is converted to *lactate*. In the fermentation of certain anaerobic organisms, *e.g., yeast, pyruvate is converted into ethyl alcohol (ethanol).* The formation of lactate is known as *lactic acid fermentation* and that of ethanol, *alcoholic fermentation* (Fig. 17.3).

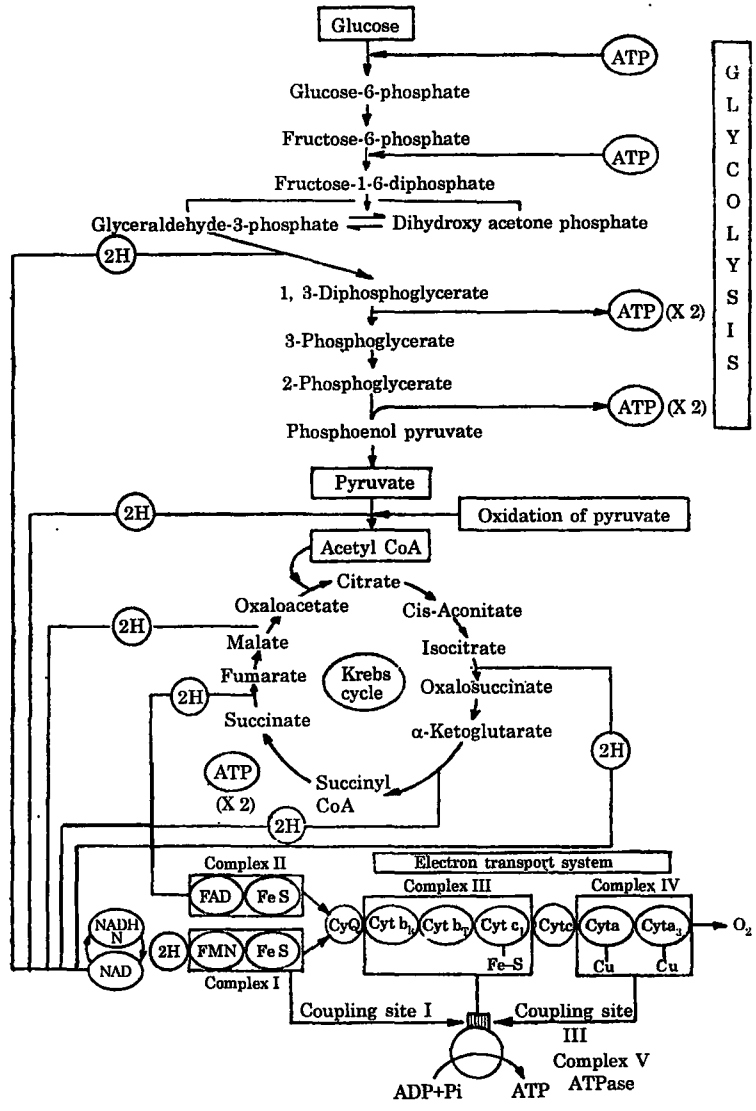


Fig. 17.3. Outline of cell respiration.

The EMP pathway is found in many microorganisms, *e.g.*, Enterobacteriaceae, Lactobacillaceae, saccharolytic clostridia and yeasts. The overall reaction of the pathway is as follows :

$$\text{Glucose} + 2\text{ATP} + 2\text{NAD}^+ \rightleftharpoons 2\ \text{Pyruvate} + 4\,\text{ATP} + 2\,(\text{NADH} + \text{H}^+)$$

Reactions of the EMP Pathway

Glycolysis is the breakdown of glucose upto the formation of pyruvic acid. Each glucose molecule forms two molecules of pyruvic acid. The breakdown takes place in a series of steps, each catalysed by a specific enzyme. At the end of the series the two pyruvic acid molecules retain most of the energy of the original glucose molecule. The reduction of glucose during glycolysis provides compounds for the synthesis of proteins, fats and nucleic acid. Most of the steps of glycolysis are reversible.

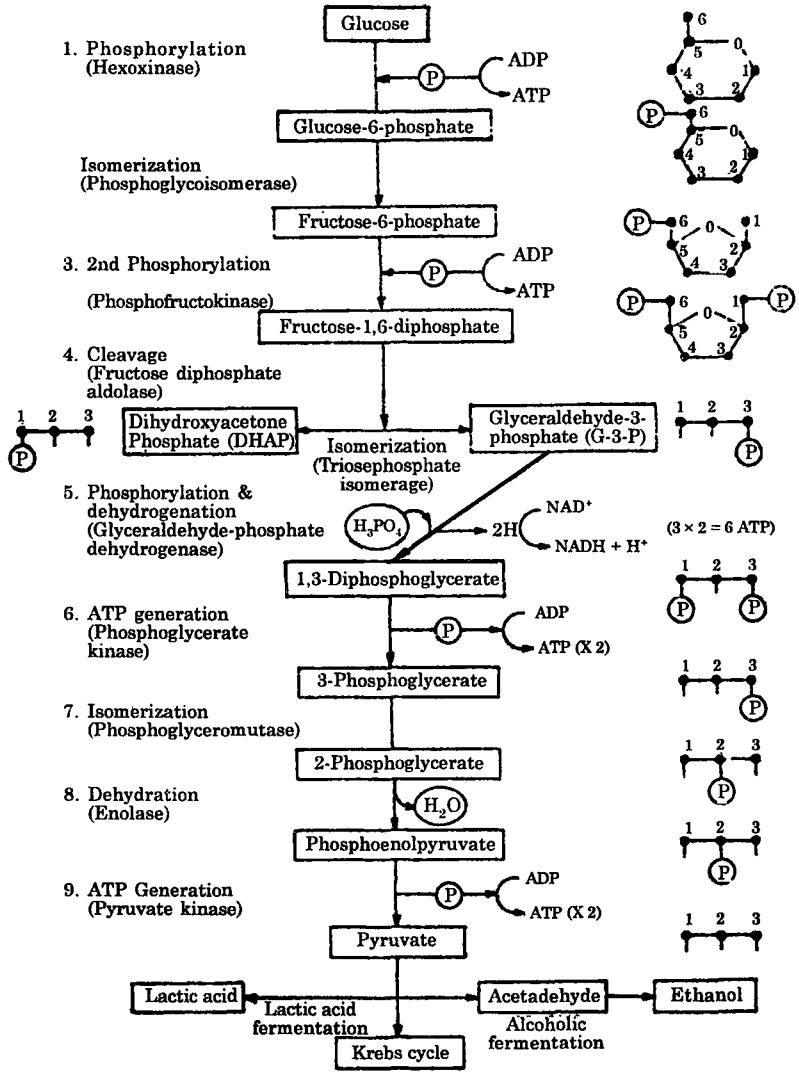


Fig. 17.4. The Embden-Meyerhof-Parnas pathway.

Glycolysis may be divided into two phases, a *preparatory* phase and an *oxidative* phase. In the following account, glycolysis has been dealt with in 9 steps, of which steps 1 to 4 represent the preparatory phase and steps 5 to 9 the oxidative phase. In the preparatory phase breakdown of glycose and *low energy phosphorylation* occurs, and energy is *expended.* In the oxidative phase *high energy* phosphate bonds are formed and energy is *stored.*

1. Phosphorylation of glucose : Glucose normally resists breakdown because it is a fairly sable compound. If its breakdown is to take place it must first be made more reactive. The 'activation' of glucose takes place by a reaction called *oxidative phosphorylation.* A phosphate group is attached to glucose by a low energy phosphate bond (–P) and *glucose-6-phosphate* is formed. The phosphate group is derived from ATP, which breaks down to ADP. The reaction is catalysed by the enzyme *hexokinase.*

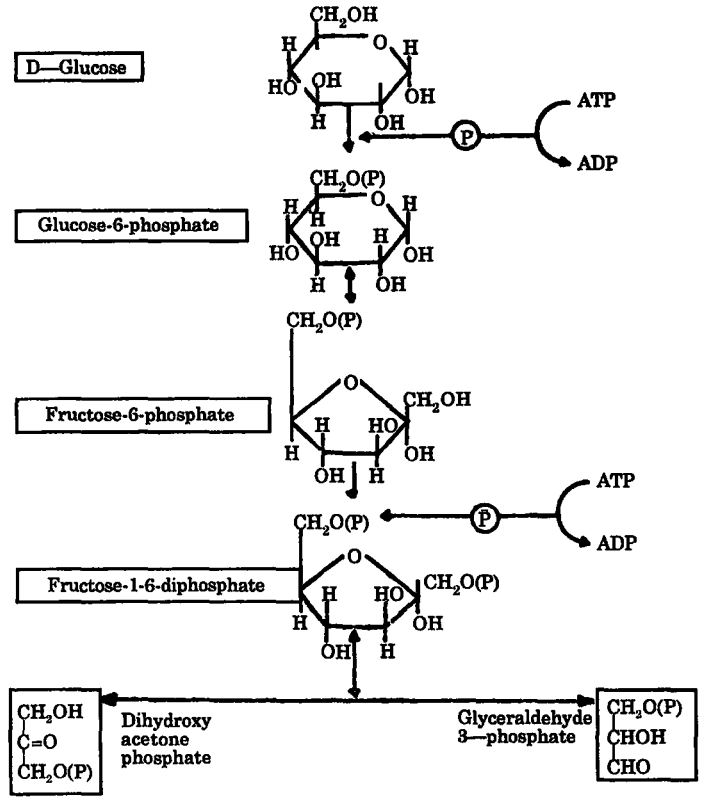


Fig. 17.5. Preparatory phase of the EMP pathway.

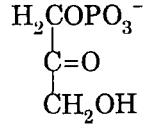
Glycogen in mammals and *starch* in plants may also be converted to glucose-6-phosphate, either directly or after first being broken down to glucose. In mammals the hormones *insulin* and *estrogen* promote phosphorylation of blood glucose to glucose-6-phosphate. *Adrenalin* has the reverse effect, bringing about dephosphorylation of glucose.

2. Isomerization of glucose 6-P to fructose-6P : The second reaction in the EMP pathway is the isomerization of *glucose 6-phosphate to fructose-6-phosphate.* This reaction is catalysed by *phosphoglucoisomerase.* Free *fructose* may also directly become fructose-6-phosphate with the help of ATP.

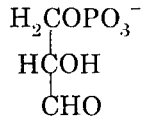
3. Second phosphorylation : In the second phosphorylation reaction *fructose-6-phosphate* is phosphorylated to *fructose-1, 6-diphosphate.* The reaction is catalysed by the enzyme *phosphofructokinase.* The high energy bond of ATP is utilized for synthesizing the low energy ester-phosphate bond of fructose-1, 6-diphosphate.

4. Cleavage of fructose-1, 6-diphosphate : Fructose-1, 6 diphosphate splits into two 3 Chalves between carbon atoms 3 and 4 under the catalytic action of the enzyme *fructose diphosphate*

aldolase. The two halves (*triose phosphates*) each contain three carbon atoms but are not identical. One half is *dihydroxyacetone phosphate* (DHAP) and the other *glyceraldehyde-3-phosphate* (*3 phosphoglyceraldehyde* : PGAL). DHAP can readily be converted to PGAL. Thus two molecules of PGAL are formed from one molecule of fructose-1, 6-diphosphate. All subsequent reactions must be considered as operating twice. The isomerization reaction is catalysed by *triose phosphate isomerase.*



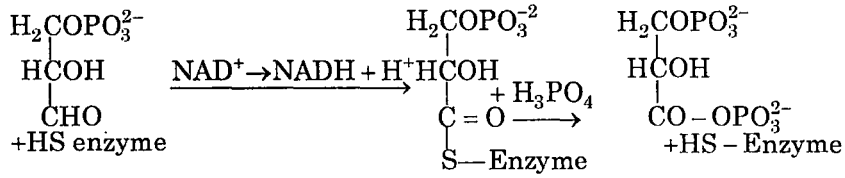
Dihydroxyacetone phosphate



Glyceraldehyde-3-phosphate

5. Phosphorylation and oxidative dehydrogenation (Fig. 17.6) : This is the first reaction in the energy yielding phase of the EMP pathway. *Glyceraldehyde-3-P* undergoes combined phosphrylation and oxidative dehydrogenation, which is catalysed by the enzyme *glyceraldehydephosphate dehydrogenase.* This enzyme is unqiue in that it can also transfer acyl groups.

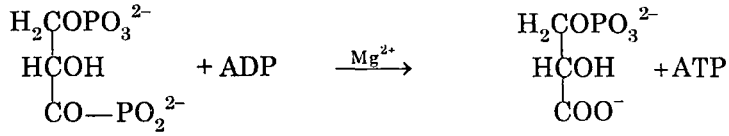
Oxidation of the glyceraldehyde-3-phosphate molecule involves removal of 2 electrons and 2 protons (the equivalent of 2H atoms). The coenzyme molecule involved in the reaction is *nicotinamide adenine dinucleotide* (NAD). The oxidized form of this molecule has a net positive charge and is hence written as NAD^+. When glyceraldehyde-3-phosphate is oxidized, NAD^+ is reduced. Two electrons and one proton are added to the nicotinamide part of the molecule and one proton (H^+) is released into the aqueous medium.



Glyceraldehyde-3-phosphate 1, 3-diphosphoglycerate (1-3-DPG)

$$NAD^+ + 2H^+ + 2e^- \rightleftharpoons NADH + H^+$$

6. Formation of ATP from 1, 3-DPGA : The high-energy phosphate group in 1, 3 diphosphoglycerate is transferred to ADP, resulting in the formation *3-phosphoglycerate* (3-PGA) and ATP. The energy removed from the high-energy phosphate group of 1, 3 DPGA is stored in the high energy bond of the terminal phosphate group of ATP. The catalysing enzyme for this reaction is *phosphoglycerate kinase* (*phosphoglyceryllkinase*).



1, 3-Diphosphoglycerate (1, 3-DPGA)

3-Phosphoglycerate (3-PGA)

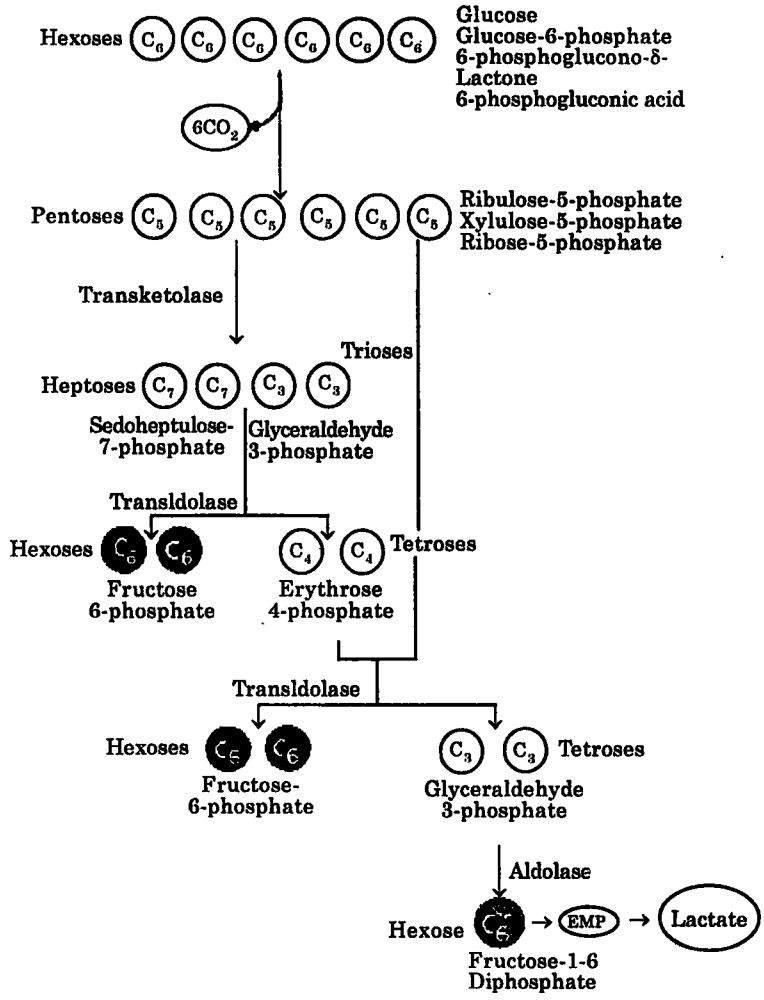
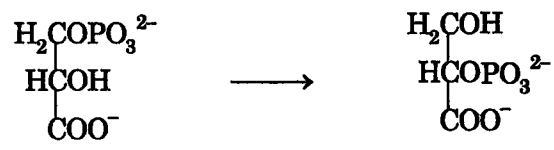


Fig. 17.6. The oxidative phase of the EMP pathway.

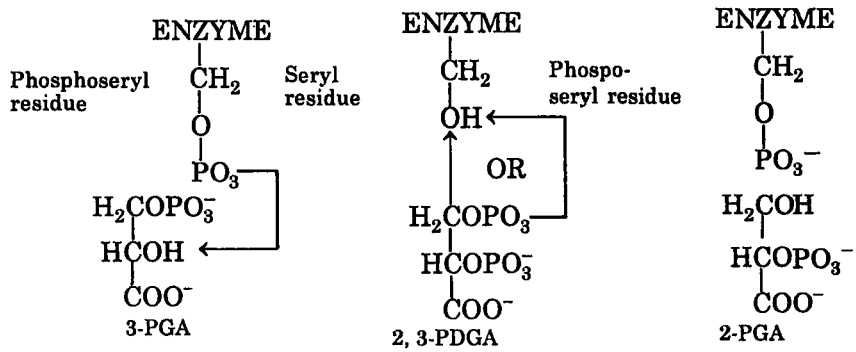
7. Isomerization : The *3-phosphoglycerate* molecule undergoes internal rearrangement to form *2-phosphoglycerate* (2-PGA). The phosphate group is transferred from the third carbon (C_3) to the second carbon (C_2). The reaction is catalysed by the enzyme *phosphoglyceromutase*. It is included in a group of enzymes called *phosphomutases* which catalyse the transfer of a phosphate group from one carbon to another carbon of the same compound.



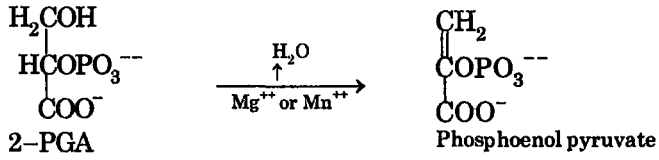
3-Phosphoglycerate 2-Phosphoglycerate

The activated form of the enzyme *phosphoglyceromutase* contains a *phosphorylated seryl residue*. The phosphate is first transferred to either *3-phosphoglycerate* (3-PGA) or *2-phosphoglycerate* (2-PGA) or from a transient intermediate 2,3-diphophoglycerate (2, 3-DPGA) on the surface of the enzyme. 2, 3-DPGA transfers either of its phosphates back to the seryl

residue. The remaining phosphate on the glycerate may be either C_2 or C_3, so that the either 2 PGA or 3-PGA (isomer) are produced. The reaction continues to bring 2 PGA and 3 PGA to equilibrium, and 3 PGA is converted to 2 PGA.



8. Dehydration of 2 PGA : The *phosphoglycerate* molecule is dehydrated by the action of the enzyme *enolase* to yield *phosphoenol pyruvic acid,* which has a high energy enolic phosphate group.



9. Formation of ATP for PEPA : In the final reaction there is transfer of phosphate from *phosphoenol pyruvic acid* to ADP to produce *pyruvic acid* and ATP. The reaction is catalysed by *pyruvate kinase.*

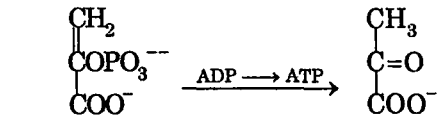


Table 17.2
EMP PATHWAY

No. Substrate	*Product*	*F° (cal/mole)*
1. Glucose	Glucose 6-P	+ 5,000
2. Glucose-6-P	Fructose-6-P	0
3. Fructose-6-P	Fructose-1, 6-dP	+ 5,000
4. Fructose-1, 6-dP	Glyceraldehyde-3-P Dihydroxyacetone-P	+ 4,000
5. Dihydroxyacetone-P	1, 3-Diphosphoglyceric acid	+ 2,000
6. 1, 3-Diphosphoglyceric acid	3-Phosphoglyceric acid	– 27,000
7. 3-Phosphoglyceric acid	2-Phosphoglyceric acid	
8. 2-Phosphoglyceric acid	Phosphoenol pyruvic acid	0
9. Phosphoenol pyruvic acid	Pyruvic acid	– 27,000

II. Hexose monophosphate (HMP) pathway (pentose phosphate or Warburg Dickens pathway) (Fig. 17.7 and 17.8)

The HMP pathway functions in the fermentation of several carbohydrates in many microorganisms, and is a 'shunt' or 'loop' in the EMP pathway. It is also found in animals, where it is important in the liver and adrenal cortex. In this pathway one molecule of *glucose-6-phosphate* is converted into one molecule of *glyceraldehyde* and three molecules of CO_2 are released. The HMP pathway yields only half the energy of the EMP pathway. It is not believed to be a major energy-yielding pathway in microorganisms. Rather, its function is to provide ribose phosphates for synthesis of nucleotides of RNA and to provide $NADPH_2$ as a source of reducing power.

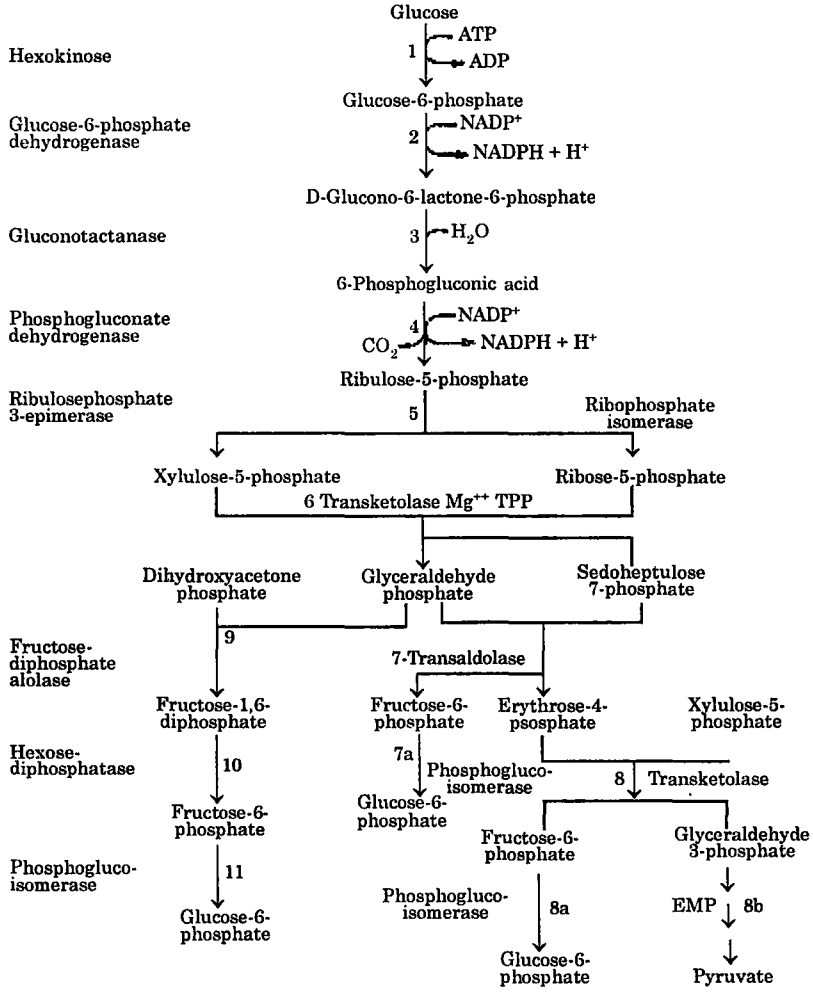


Fig. 17.7. The hexose monophosphate (HMP) pathway.

The reduction of $NADPH_2$ takes place at two stages : (*i*) Oxidation of *glucose-6-phosphate* to *6 phosphogluconate* and (*ii*) oxidation of *6 phosphogluconate to ribose 5-phosphate.*

The reactions of the HMP pathway are as follows :

(1) Glucose is phosphorylated by a phosphate group obtained from the breakdown of ATP to ADP to form *glucose-6-phosphate.* This reaction is identical to that of the EMP pathway.

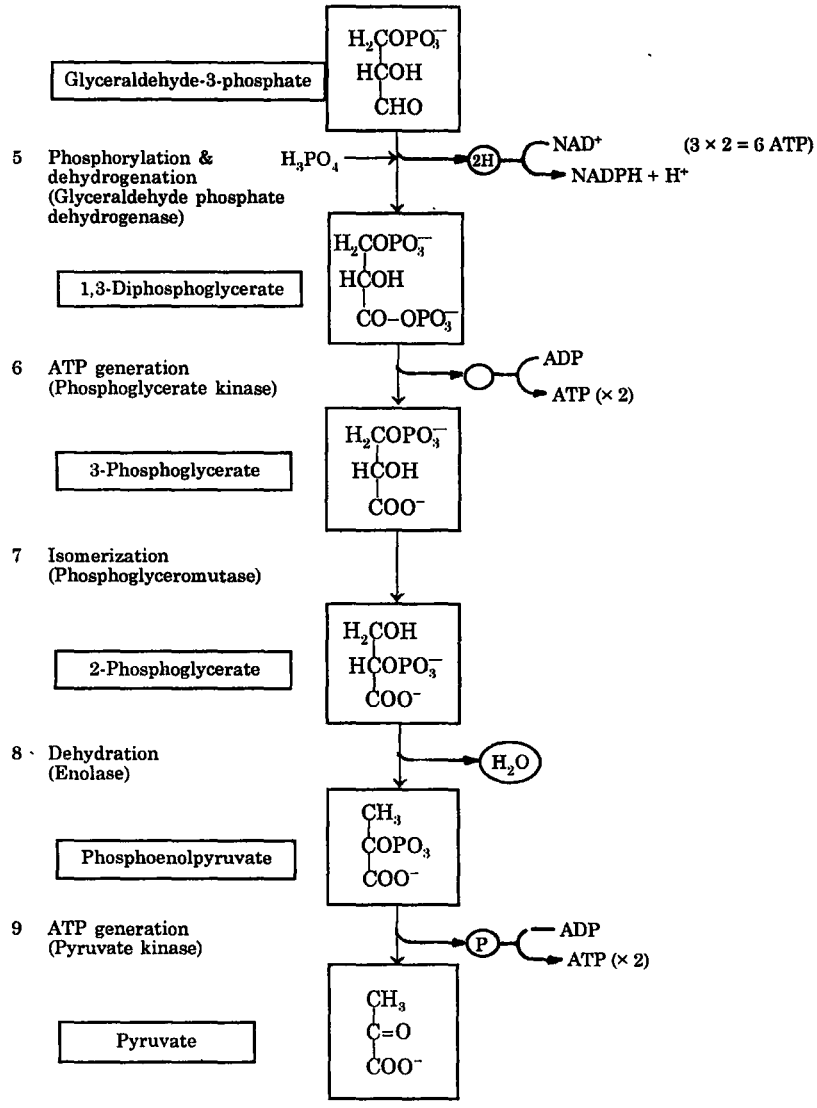


Fig. 17.8. HMP pathway : balance of carbons.

(2) Glucose-6-phosphate is oxidized to the δ-*lactone of phosphogluconic acid* (D glucose-δ-lactone 6-phosphate) by an NADP-linked glucose-6-*phosphate dehydrogenase.*

(3) The δ-lactone of phosphogluconic acid is immediately hydrolysed to *6-phosphogluconic acid by gluconolactonase* (D glucono δ-*lactone hydrolase*).

(4) 6-Phosphogluconic acid is simultaneously *decarboxylated* and *oxidized* to form *D ribulose-5-phosphate.* $NADP^+$ accepts the hydrogens and is reduced to NADPH + H^+. The reaction is catalysed by *phosphogluconate dehydrogenase.* The fourth reaction coverts the hexose into a pentose.

(5) Ribulose 5-phosphate is acted upon by two different enzymes. *Ribulosephosphate-3 epimerase* coverts ribulose 5-phosphate to *xylulose 5-phosphate,* while *ribosephosphate isomerase* coverts it to *ribose 5-phosphate.* Ribose 5-phosphate is a precursor for purine, pyrimidine and aromatic amino acid biosynthesis.

(6) *Xylulose-5-phosphate* and *ribose-5-phosphate* (C5) form *sedoheptulose-7-phosphate* (C7) and *glyceraldehyde-3-phosphate* (C3). Thus two *pentose* phosphate (C5) molecules react to give a *heptose* phosphate (C7) and a *triose* phosphate (C3).

C5	+	C5	⇌	C7	+	C3
Pentose		Pentose		Heptose		Triose

The reaction is catalysed by *transketolase* in the presence of cofactors *thiamine pyrophosphate* (TPP) and Mg^{++}.

(7) *Sedoheptulose phosphate* reacts with *glyceraldehyde 3-phosphate* to form *fructose-6-phosphate* and *erythrose-4-phosphate*. This *transaldolation reaction* is catalysed by *transaldolase*. The reaction is reversible. Erythrose-4-phosphate is an important precursor for purine, pyrimidine and aromatic amino acid biosynthesis.

C7	+	C3	⇌	C6	+	C4
Heptose		Triose		Hexose		Tetrose

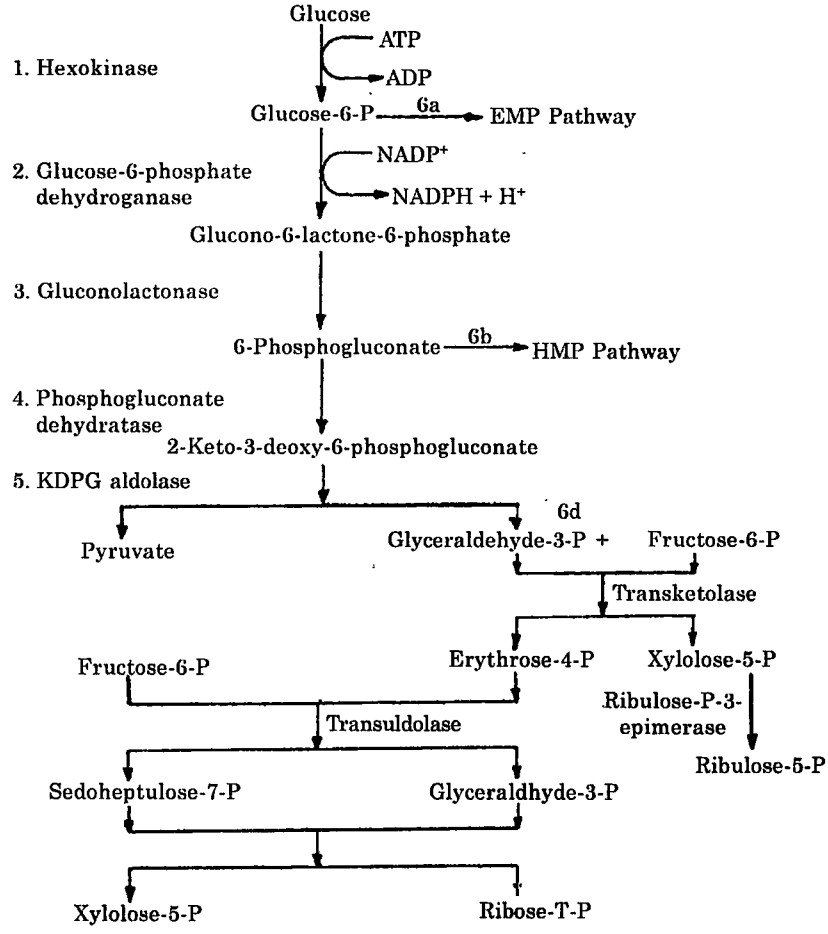


Fig. 17.9. The Entner-Duodoroff (ED) pathway.

Ribose-5-phosphate may also be the acceptor, in which case *octulose-8-phosphate* may be formed.

(8) *Erythrose-4-phosphate* accepts a 2-carbon unit from *xylulose-5-phosphate* to form *fructose 6-phosphate* and *glyceraldehyde-3-phosphate.* This reaction is also catalysed by a *transketolase* as in reaction (6). The *fructose-6-phosphate* (8*a* and 7*a*) and the *glyceraldehyde-3-phosphate*) (8*b*) of the HMP pathway link up with the EMP pathway. *Fructose-6-phosphate* can be converted to *glucose-6-phosphate* with a *glucosephosphate isomerase* (*phosphoglucoisomerase*). Glyceraldehyde-3-phosphate can also follow the reverse EMP pathway to form *glucose-6-phosphate.*

(9) Reactions (9), (10) and (11) are the reverse of those in the EMP pathway. The formation of dihydroxyacetone phosphate by *triosephosphate isomerase* is identical to the reactions in the EMP pathway. The two triose phosphate molecules condense to form *fructose 1, 6 disphosphate.* This reaction is identical to reaction (4) of the EMP pathway and is catalysed by *fructose disphosphate aldolase.*

(10) The conversion of *fructose-1, 6-diphosphate* to *fructose 6-phosphate* requires a separate enzyme, *hexosediphosphatase,* because *phospho-fructokinase* which catalyses reaction (3) in the EMP pathway cannot do so in the reverse direction.

(11) *Fructose-6-phosphate* gives rise to *glucose-6-phosphate* by the action of *phosphoglucoisomerase,* the enzyme which catalyses reaction (2) in the EMP pathway.

III. The Entner-Duodoroff (ED) pathways (Figs. 17.9 and 17.10)

This pathway was discovered by Entner and Duodoroff (1952) in the course of metabolic studies on *pseudomonas saccharophilia,* and has been since then found in many other species of this genus. The first three reactions are identical to those of the HMP pathways. It is, however, not certain whether or not the enzymes differ in their kinetic characteristics.

(1) *Glucose* is first phosphorylated by ATP to *glucose-6-phosphate,* the catalysing enzyme being *hexokinase.*

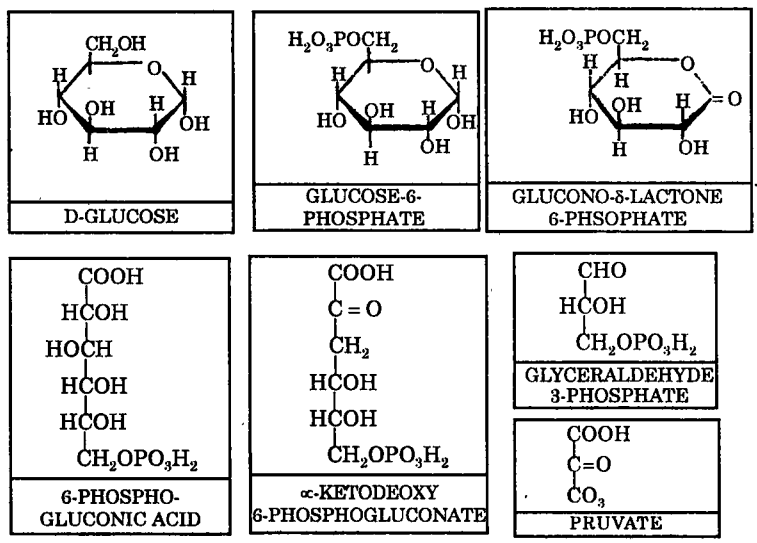


Fig. 17.10. The ED pathway : structure of some compounds.

(2) Glucose-6-phosphate is oxidized with NADP-linked *glucose-6-phosphate dehydrogenase* to produce the δ-lactone of phosphogluconic acid (*6-phosphoglucono-δ-lactone*).

(3) 6-Phosphoglucono-δ-lactone is immediately hydrolysed to 6-*phosphogluconic acid* by *gluconolactonase* (*D-glucono-δ-lactone hydrolase*).

(4) 6-Phosphogluconic acid is hydrolysed by a *phosphogluconate dehydratase* to form an α-*ketodeoxy sugar phosphate* (2-*keto-3-deoxy-6-pho*˜*phogluconate* : *KDPG*).

(5) The α-ketodeoxy sugar phosphate is cleaved by an *aldolase* type enzyme (*KDPG-aldolase*) to *pyruvate* and *glyceraldehyde*-3-*phosphate*. This reaction is very similar to the cleavage of fructose-1, 6-diphosphate in the EMP pathway. KDPG also catalyses the enolization of pyruvate.

(6) The metabolites of the ED pathway can lead to other glycocytic pathway :

(a) Glucose-6-phosphate can follow the EMP pathway to yield fructose-6-phosphate ⟶ fructose-1, 6-diphosphate ⟶ dihydroxyacetone phosphate + glyceraldehyde.

(b) 6-Phosphogluconic acid can lead to the HMP pathway.

(c) Glyceraldehyde-3-phosphate is free to use the EMP pathway to pyruvate.

(d) The importance of the pathway lies in the fact that the organisms can produce pentose precursors leading to pyridine and pyrimidine biosynthesis, as well as the biosynthesis of aromatic amino acids by a reverse HMP pathway. *Glyceraldehyde* can condense with *fructose-6-phosphate* to yield *erythrose-4-phosphate* and *xylulose-5-phosphate,* the reaction being catalysed by a *transketolase. Xylulose-5-phosphate* is converted into *ribulose-5-phosphate* by *ribulose phosphate-3-epimerase. Erythrose-4-phosphate* and *fructose-6-phosphate,* under the catalytic action of an *aldolase,* form *sedoheputlose-7-phosphate* and *glyceraldehyde-3-phosphate.* A second *transketolase* catalyses the formation of *xylulose-5-phosphate* and *ribose-5-phosphate* from the above two compounds.

IV. Phosphoketolase pathways

The heterofermentative lactobacilli and the Bifidobacteria possess the *phosphoketolase* pathway, which is a variation of the HMP pathway. There are two types of phosphoketolase pathways, the *pentose phosphoketolase pathway* and the *hexose phosphoketolase pathway.*

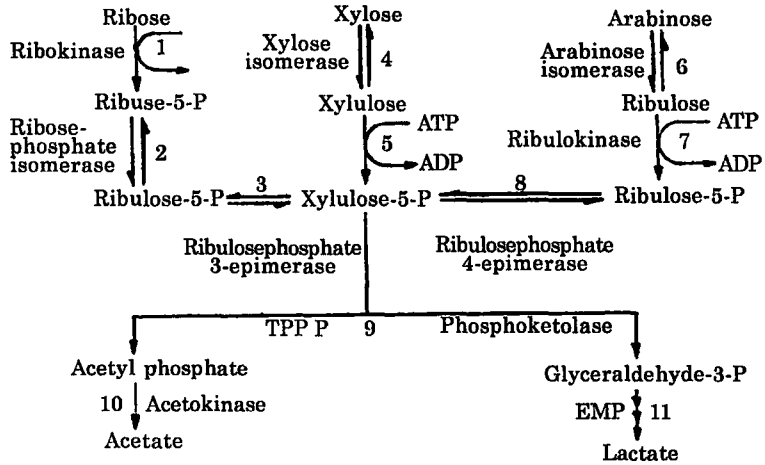


Fig. 17.11. The pentose phosphoketolase pathway.

1. Pentose Phosphoketolase pathway (Fig. 17.11)

The carbon source is ribose as well as other pentoses. Ribose-5-phosphate or xylulose-5-phosphate are formed *via* the HMP pathway. The pentose phosphoketolase pathway is found in

leuconostoc mesenteroides and *Leuconostoc plantarum,* in which the EMP, HMP and ED pathways are absent.

(1) *Ribokinase* transfers a phosphate group from ATP to ribose, yielding *ribose-5-phosphate.*

(2) Ribose-5-phosphate is isomerized to *ribulose-5-phosphate* by *ribosephosphate isomerase.*

(3) *Ribulose phosphate-3-epimerase* converts ribulose-5-phosphate to *xylulose-5-phosphate.*

(4) All other pentoses are also converted to *xylulose-5-phosphate. Xylose* is isomerized to *xylulose* by *xylose isomerase.*

(5) Xylulose is phosphorylated by ATP under the catalytic action of *xylulose linkase* to *xylulose-5-phosphate.*

(6) *Arabinose* is isomerized to *ribulose* by *arabinose isomerase.*

(7) *Ribulose* is phosphorylated by ATP to *ribulose-5-phosphate by ribulokenase.*

(8) *Ribulose-5-phosphate* is converted to *xylulose-5-phosphate* by *ribulosephosphate-4-epimerase.*

(9) Xylulose-5-phosphate plays a key role in the pentose phosphoketolase pathway, because the key enzyme of the pathway, *phosphoketolase,* reacts only with this compound. The phosphoketolase splits xylulose-5-phosphate into an *acetyl phosphate* and *glyceraldehyde-3-phosphate.* The reaction requires *thiamine pyrophosphate* (TPP) and *inorganic phosphate* (Pi).

(10) *Acetyl phosphate* is converted to *acetate* by *acetokinase.*

(11) *Glyceraldehyde* is metabolized via the EMP pathway to *pyruvate* and finally *lactate.*

When glucose is the substrate, it is metabolized to ribulose-5-phosphate via the HMP pathway. Further metabolism is by the pentose phosphoketolase pathway.

$$\underset{\text{Glucose}}{\overset{1}{\longrightarrow}}\ \underset{\text{Glucose-6-P}}{\overset{2}{\longrightarrow}}\ \underset{\text{6-Phosphogluconic acid}}{\overset{CO_2\ 3\uparrow}{\longrightarrow}}\ \underset{\text{Ribulose-5-P}}{}$$

$$\text{ATP} \longrightarrow \text{ADP}\quad \text{NAD}^+ \longrightarrow \text{NADH}^+ + \text{H}^+\quad \text{NADH}^+ \longrightarrow \text{NADH} + \text{H}^+$$

2. Hexose Phosphoketolase Pathway (Fig. 17.12)

This pathway is found in the genus *Bifidobacterium* (*Lactobacillus bifidus*) which lacks *glucose-6-phosphate dehydrogenase* and *fructose-diphosphate aldolase.* Because of this, the EMP, HMP, ED or pentose phosphoketolase pathway cannot operate.

The key reaction is the cleavage of *fructose-6-phosphate* into *erythrose-phosphate* and *acetyl phosphate by phosphoketolase.*

In a reverse HMP pathway involving a *transaldolase* and a *transketolase,* the compound *xylulose-5-phosphate* is formed.

This is splitted into *glyceraldehyde-3-phosphate* and *acetyl phosphate,* as the pentose phosphoketolase pathway.

(B) Aerobic Pathways of Pyruvate Metabolism

Pyruvic Acid Oxidation

Pyruvic acid, the end product of glycolysis, does not enter the citric acid cycle directly. The 3-carbon atom molecule of pyruvic acid is first changed into a 2-carbon atom *acetic acid* molecule. One carbon is released as carbon dioxide (*decarboxylation*). Acetic acid on entering the

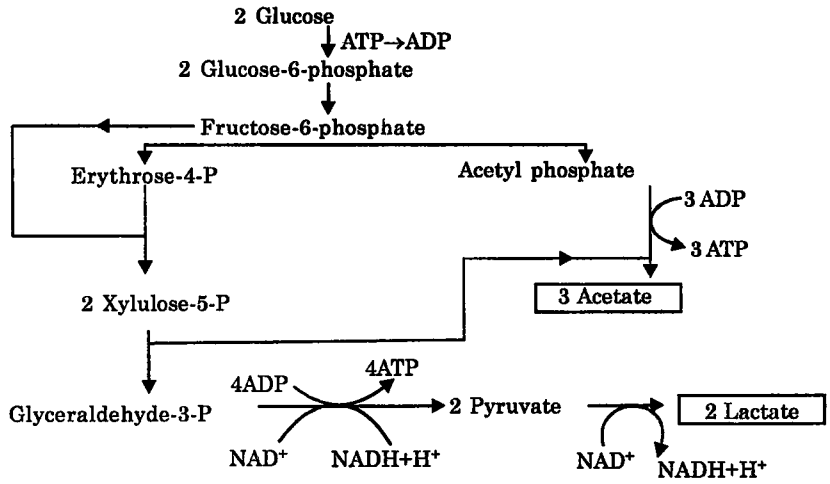


Fig. 17.12. The hexophotoketolase pathway.

mitochondria unites with *conenzyme A* (CoA) to form *acetyal CoA* ('active acetate'). This reaction is catalysed by several enzymes collectively known as *pyruvate dehydrogenase.* Two hydrogen atoms are released in the reaction. They are accepted by NAD^+ to form NADH + H^+. The pair of hydrogen atoms is passed down the electron transport system, and three molecules of ATP are generated. Since two pyruvic acid molecules are formed during metabolism of one molecule of glucose, in all six molecules of ATP are formed during oxidation of the two pyruvic acid molecules.

I. Krebs Citric Acid Cycle (Figs. 17.13 and 17.14)

It has been seen that pyruvate is converted either into *ethyl alcohol* (alcoholic fermentation) or *lactate* (lactic acid fermentation or muscle glycolysis) in the absence of oxygen (*anaerobic condition*). In the presence of oxygen (*aerobic condition*), however, its metabolism is different. Pyruvate is oxidized to *acetyl CoA,* which then undergoes a series of changes referred to as *Krebs citric acid cycle.* The citric acid cycle is also called the *tricarboxylic acid cycle* (TCA cycle), since citric acid is a tricarboxylic acid, *i.e.,* with three COOH groups. Each cycle results in the conversion of one molecule of acetic acid, in the form of acetyl CoA to carbon dioxide and water.

1. Formation of citrate : The first step in the citric acid cycle is the condensation of the 2-carbon atom *acetyl-CoA* with a 4-carbon atom *oxaloacetate* molecule to form a 6 carbon atom molecule of *citrate.* This reaction is irreversible. Acetyl CoA gives its acetyl group to oxaloacetate to form the citrate molecule. CoA is released, and reacts with more pyruvate. The linkage between the acetyl group and CoA in acetyl CoA is hydrolysed by a molecule of water. This catalysing enzyme is *citrate synthetase.*

2. Dehydration : Citrate undergoes dehydration under the catalytic action of the enzyme *aconitase to form cis-aconitate* (6 carbon atoms).

3. Hydration I : Cis-aconitate is rehydrated to *isocitrate,* again under the influence of *aconitase.* The transformation of citrate to isocitrate is one of *isomerization.* Citrate can also yield isocitrate without cis-aconitate occurring as a free intermediate.

4. Dehydrogenation I : Isocitrate undergoes *dehydrogenation* in the presence of the enzyme *isocitrate dehydrogenase* to form *oxalosuccinate.* The pair of hydrogen atoms removed is accepted by NAD^+ to form NADH + H^+. The occurrence of oxalosuccinate has been doubted by some workers. It may occur only as an enzyme bound-intermediate.

5. Decarboxylation I : Oxalosuccinate (or isocitrate) undergoes *decarboxylation* to form *α-ketoglutarate.* One CO_2 is removed. Because of the loss of one carbon atom during *decarboxylation,* the α-ketoglutarate molecule has five carbon atoms. The reaction is catalysed by *carboxylase.*

6. Dehydrogenation II and Carboxylation II : α-Ketoglutarate undergoes simultaneous *decarboxylation* and *dehydrogenation,* and joins with coenzyme A to form *succinyl-CoA,* a 4 carbon atom derivative of coenzyme A. The reaction is catalysed by an *α-ketoglutarate dehydrogenase* complex involving four coenzymes, at least three enzymes, and magnesium ions. The hydrogen atoms released are accepted by NAD^+ to form NADH + H^+. The hydrogens are passed down the hydrogen transport system, and three molecules of ATP are generated.

7. Phosphorylation of ADP : Succinate is released from succinyl CoA under the catalytic action of *succinic thiokinase.* CoA is released in this reaction. The reaction requires GDP (*guanosine diphosphate*) or IDP (*inosine diphosphate*). In the presence of inorganic phosphate, GDP or IDP undergoes phosphorylation to form either GTP (*guanosine triphosphate*) or IDP (*inosine triphosphate*). In the citric acid cycle this is the only example of the generation of a high energy phosphate at the substrate level. ATP may be formed from either GTP or ITP by means of a *phosphokinase.* The entire reaction may be summarized as follows :

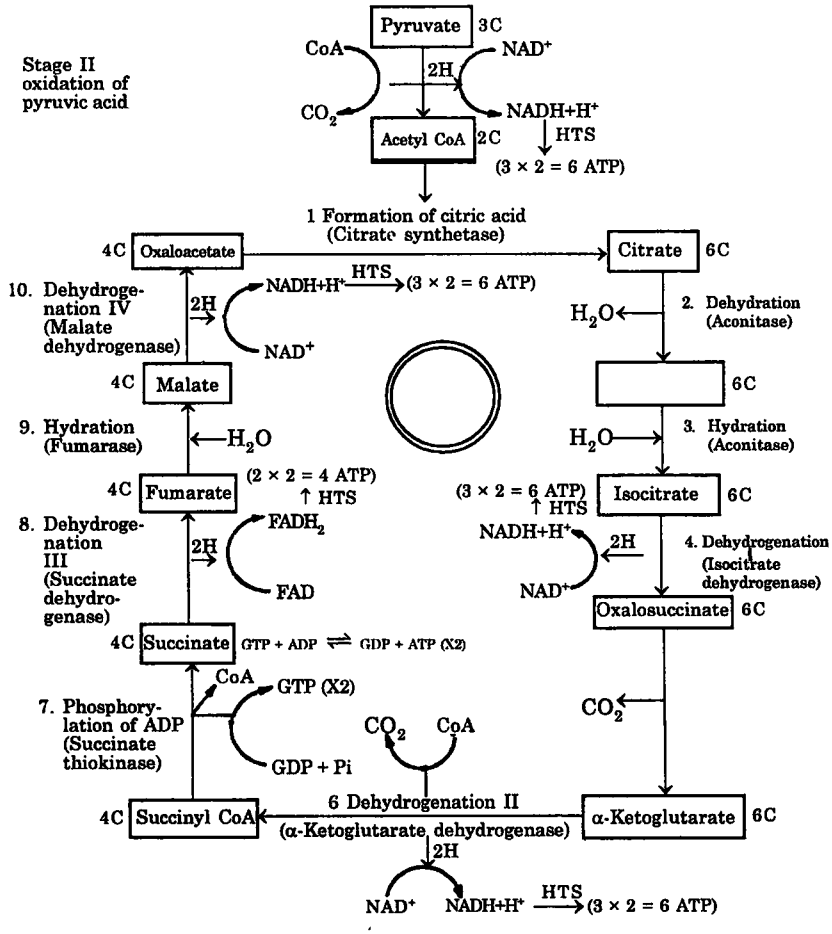


Fig. 17.13. Krebs citric cycle. The names of the enzymes catalysing each step are given in brackets.

(*i*) GDP + Pi ⟶ GTP *or* IDP + Pi ⟶ ITP

(*ii*) GTP + ADP ⟶ GDP + ATP *or* ITP + ADP ⟶ IDP + ATP

8. Dehydrogenation III : Succinate undergoes *dehydrogenation* under the catalytic action of *succinate dehydrogenase* to form *fumarate.* The pair of hydrogen atoms formed is transferred directly to *flavoprotein,* without the participation of NAD^+.

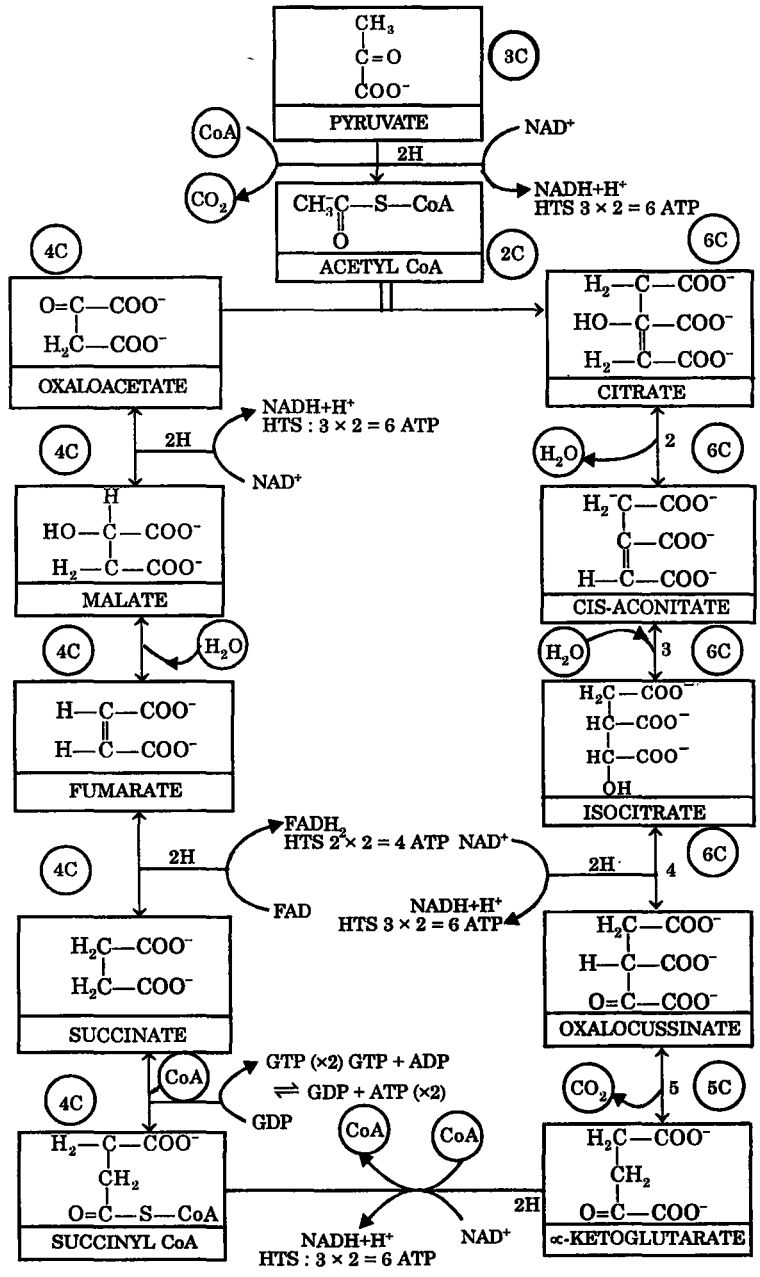


Fig. 17.14. Krebs citric acid cycle : Structure of intermediates.

Flavoprotein contains FAD (flavin adenine dinucleotide) and nonhaeme iron (NHI). FAD accepts the hydrogen atoms to form $FADH_2$. Because the first ATP-generating step of the hydrogen transport system is passed over, only *two* molecules of ATP are generated per pair of hydrogen atoms when FAD is the hydrogen acceptor.

9. Hydration II : Fumarate is hydrated to form *malate.* The reaction is catalysed by the enzyme *fumarase.*

10. Dehydrogenation IV : In the final reaction malate is transformed into *oxaloacetate,* which is thus regenerated. The catalytic enzyme is *malate dehydrogenase.* Dehydrogenation takes place during the process. NAD^+ accepts the hydrogen atoms to form $NADH + H^+$. The pair of hydrogens pass down the hydrogen transport system, and three molecules of ATP are generated.

It should be noted that two molecules of pyruvate are formed per molecule of glucose during glycolysis. Therefore, two molecules of oxaloacetate per molecule of glucose are formed at the end of Krebs cycle. The fate of only one pyruvate molecule has been traced in the figure.

The essential features of the citric acid cycle may now be summarized :

1. The starting point for the citric acid cycle is acetyl CoA and oxaloacetate.

2. Acetyl-CoA is formed from the products of metabolism of carbohydrates (pyruvate), fats (fatty acids) and proteins (certain amino acids).

3. Oxaloacetate is a normal constitutent of cells. It reacts with acetyl-CoA and water to form *citrate.*

4. Citrate undergoes a series of degradations to form oxaloacetate, which re-enters the cycle.

5. In each cycle two carbon atoms are released as carbon dioxide (*decarboxylation*), most of which is lost as a waste product.

6. Four *dehydrogenation* reactions occur in each cycle, releasing four pairs of hydrogen atoms. There pairs of hydrogens are accepted by NAD^+ to form $NADH + H^+$. Each pair of hydrogens when transported through the hydrogen transport system generates *three* molecules of ATP. Thus nine molecules of ATP are generated by hydrogens and accepted by NAD^+. One pair of hydrogens is accepted by FAD of flavaprotein to form $FADH_2$. These hydrogens, when passed through the hydrogen transport system, yield only *two* molecules of ATP, since the first ATP-generating step is passed over. Thus in each Krebs cycle proper, 11 molecules of ATP are generated through the hydrogen transport system.

7. One molecule of ATP is generated directly at the substrate level (step 7) in each cycle.

II. Electron Transport System

1. Eukaryote Electron Transport

1. In eukaryotes the electron transport chain and the enzymes of (Figs. 17.14 and 17.15) the Krebs TCA cycle are found in the mitochondria. The Krebs cycle enzymes are located in the *matrix* of the mitochondrion, while the components of the electron transport chain are located in the *inner membrane.*

2. A number of dehydrogenation reactions take place during cell respiration : *one* during *aerobic glycolysis, one* during *oxidation of pyruvic acid* and *four* during each turn of the *Krebs cycle.*

3. The hydrogen (2H) acceptor in all but one of these reactions is *nicotinamide adenine dinucleotide* (NAD). The hydrogens released during the reduction of *succinate* to *fumarate* in the Krebs cycle are accepted by *flavin adenine dinucleotide* (FAD).

4. The redoxidation of NAD/FAD takes place by the passage of electrons through a series of *intermediate carriers.* These are *flavin mononucleotide* (FMN) of flavoprotein, *coenzyme* Q (CoQ)

or *ubiquinone* (UQ), and *cytochromes* b_K, b_T c_1, c and a a_3. As the electrons pass down the chain there is alternate *reduction* and *oxidation* of its components.

5. The carriers are arranged in a series of gradually increasing *redox potentials*. Electrons tend to pass from a carrier with a more negative redox potential to the carrier with a more positive one.

6. For each pair of electrons passing down the electron transport system, *three molecules of ATP are generated,* one at each of the three sites in the chain. These are the sites of oxidative phosphorylation.

Site I : between NAD and CoQ.

Site II : between cytochrome *b* and cytochrome *c*.

Site III : between cytochrome *c* and oxygen.

7. The final hydrogen acceptor is molecular *oxygen,* and it is because of this that most organisms require oxygen in order to survive.

8. The entire respiratory chain and the three sites of ATP generation can be represented thus :

$$\text{NAD} \xrightarrow[\text{ATP}]{} \text{FMN. Fe-S-CoQ. } c_Y t b_K . c_Y t\ b_T \xrightarrow[\text{ATP}]{} c_Y t c l . c_Y t c \xrightarrow[\text{ATP}]{} c_Y t\ a . a_3 \longrightarrow o_2$$

9. When FAD is the initial acceptor, the first ATP generating step is passed over. Consequently only *two* molecules of ATP are generated per pair electrons psssing down the respiratory chain.

(C) Fermentation

Respiration is an ATP generating process in which *either inorganic or organic compounds* function as *electron donors* and *inorganic compounds* serve as the ultimate *acceptors*. The terms *aerobic respiration, anaerobic respiration* and *fermentation* are often used in discussions on energy-yielding metabolism.

Aerobic respiration takes place in the presence of *free oxygen*, which is the final electron acceptor. Most organisms are *strict aerobics*. They are totally dependent on oxidative phosphorylation and cannot survive in the absence of oxygen.

In *anaerobic respiration* energy is obtained from oxidation in which *inorganic compounds* function as final electron acceptors. The ultimate electron acceptors are compounds like sulphates, nitrates and carbonates. Although these compounds contain oxygen, it is not present in the *free* form. Some microorganisms utilize oxidative phosphorylation when possible, but can also survive in the absence of oxygen. They generate ATP either by anaerobic respiration with nitrate or by fermentation. Such organisms are called *facultative anaerobes*. They burn fuels when oxygen is present, but can resort to anaerobic respiration in the absence of oxygen. Other microorganisms are *strict anaerobes* and cannot survive in the presence of oxygen because they lack the enzyme *superoxide dismuatase*.

In the strict sense the term *fermentation* refers to those energy-yielding pathways in which *organic compounds* act as both electron donors and electron acceptors. When the compounds donate electrons they become oxidized, and when they accept electrons they become reduced. During fermentation microorganisms obtain energy from organic compounds without utilizing oxygen. The compounds which serve as electron donors and electron acceptors are usually two different metabolites derived from a single substrate such as sugar. The substrate produces a mixture of end products, some of which are more oxidized and some more reduced. Thus an oxidation-reduction balance is maintained, and the average oxidation levels of the end products is the same as that of the substrate.

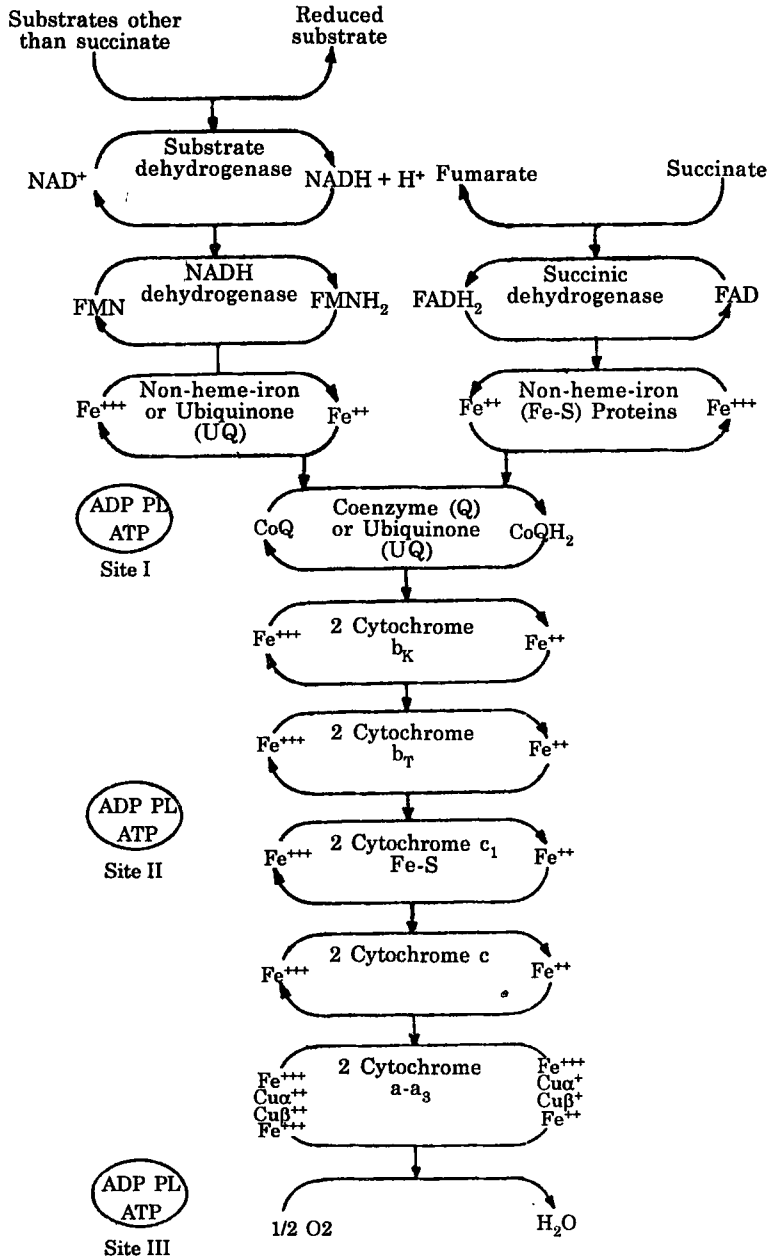


Fig. 17.15. The hydrogen/electron transport system.

The principal substrates of fermentation are carbohydrates. Bacteria can also utilize compounds like organic acids, amino acids, purines and pyrimidines.

The process of fermentation takes place in two states :

1. *Glucose* is broken down to *pyruvate* with the release of two pairs of hydrogen atoms.

2. Pyruvate or compounds derived from pyruvate are reduced by the hydrogens released in the first stage.

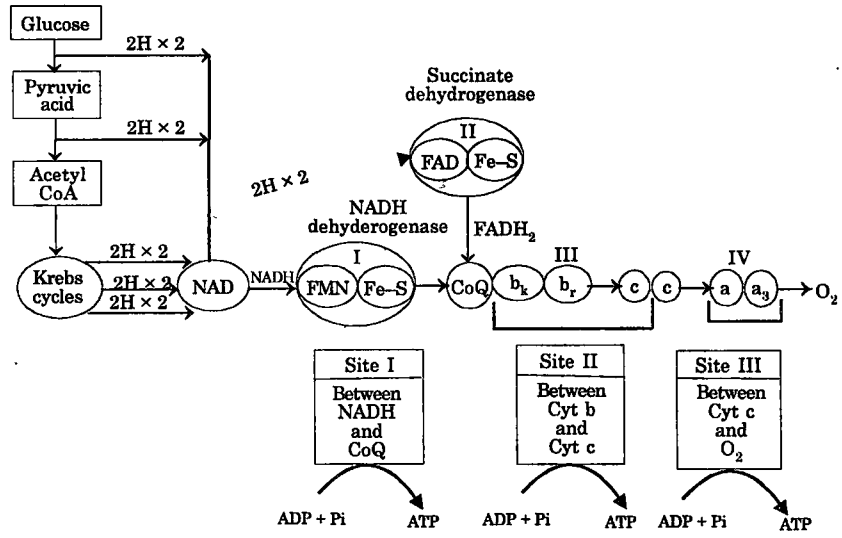


Fig. 17.16. Outline of glycolysis, pyruvic acid oxidation, Krebs cycle and oxidative phosphorylation in the electron transport system.

I. Alcoholic Fermentation

In alcoholic fermentation pyruvate is converted to *ethanol* and *carbon dioxide*. This process is characteristic of yeasts, particularly strains of *Saccharomyces cerevisiae*. It is also found in some moulds and in the Mucorales, but is comparatively rare in bacteria. In the bacterium *pseudomonas*, pyruvate is produced through the Entner-Duodoroff pathway. It is then metabolized to *ethanol* through *acetaldehyde*.

A molecule of *glucose* yields two molecules of pyruvate through the EMP pathway. Pyruvate metabolism takes place in two steps.

1. *Pyruvate* is first decarboxylate, yielding *acetaldehyde* and *carbon dioxide*. The reaction is catalysed through the enzyme *puruvate decarboxylase*, with *thiamine pyrophosphate* (TPP) as the coenzyme.

2. *Acetaldehyde* is then reduced to *ethanol* by NADH + H^+ ($NADH_2$) and NAD^+ is regenerated. The catalysing enzyme is *alcohol dehydrogenase*.

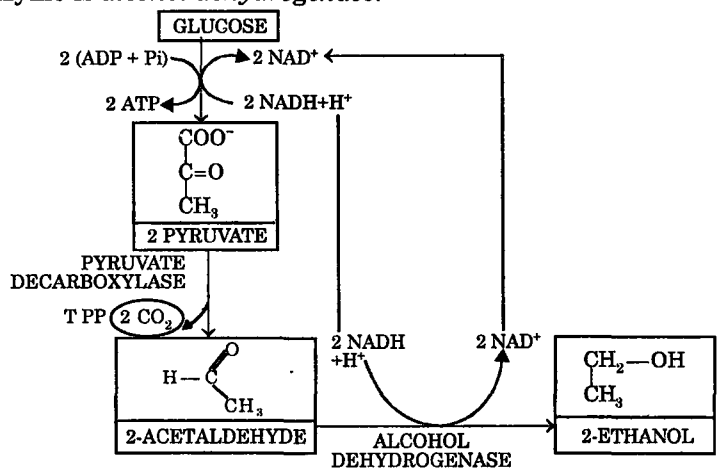


Fig. 17.17. Alcoholic fermentation in yeasts.

It will be seen that the hydrogens removed during glucose metabolism are accepted by NAD, which is reduced to $NADH_2$. It is essential that NAD is regenerated so that it can pick up more hydrogens. If this did not happen glycolysis would stop, resulting in the death of the organism.

Regeneration of NAD takes place when *acetaldehyde* is reduced to *ethanol.* Alcoholic fermentation is of economic importance in the production of beverages and in raising bread.

II. Lactic Acid Fermentation

Lactic acid fermentation is a one-step reaction similar to glycolysis of mammalian cells. *Pyruvic acid is* reduced to *lactic acid,* the reaction being catalysed by *pyruvate reductase.* Lactic acid fermentation is characteristic of the lactic acid bacteria (Lactobacillaceae) which cause spoilage of food. Although morphologically heterogeneous, the bacteria are characterized by the fact that they produce *lactic acid* as the end product.

The lactobacilli are divided into two groups, *homofermentative* and *heterofermentative* strains. The demarcation between the two groups is indefinite in some cases.

1. Homolactic fermentation : Homolactic fermentation is found in members of the genera *Streptococcus, Leuconostoc, Pediococcus* and *lactobacillus.* It is also found in muscle cells of animals. In this type of fermentation the predominant product is *lactic acid.* Other products are found only in traces.

Glucose yields two molecules of *pyruvate* through the EMP pathway. Pyruvate is reduced to *lactate* by the enzyme *lactate dehydrogenase* (*pyruvate reductase*). $NADH_2$ is the hydrogen donor and is oxidized to NAD. There is a net yield of two molecules of ATP per molecule of hexose metabolized.

$$\text{Glucose} \xrightarrow{\text{EMP}} \underset{\text{Puruvic acid}}{CH_3CO\,COOH} \xrightarrow[NADH_2 \longrightarrow NAD]{} \underset{\text{Lactic acid}}{CH_3CHOH.COOH}$$

In addition to glucose, homolactic microorganisms also ferment other monosaccharides like *fructose, mannose,* and *galactose* and disaccharides like *lactose, maltose* and *sucrose.* These sugars are apparently converted into the intermediates of the EMP pathway by inducible enzymes.

2. Heterolactic fermentation : (Figs. 17.18 and 17.19). This type of fermentation is found in some strains of *Leuconostoc, Lactobacillus,* the anaerobic peptostreptococci and the anaerobic species of *Eubacterium, Ramibacterium, Bifidobacterium* and *Catenabacterium.* In heterolactic fermentation products like *ethanol, glycerol, acetate, propionic acid,* CO_2 and *butyric acid* are found in addition to lactic acid. Among the heterofermenters are included organisms that produce less than 1.8 moles of lactic acid per mole of glucose and, in addition, some of the compounds mentioned above. In *Leuconostoc type* heterofermentation, *acetate, ethanol* and *glycerol* are formed in addition to lactic acid. In *Peptostreptoccocus type* heterofermentation, *lactic* and *propionic* acids are the end products of glucose fermentation. In a third type of heterofermentation, *lactic* and *butyric* acids are the end products.

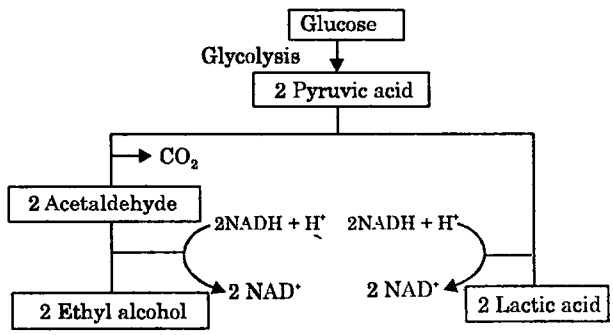


Fig. 17.18. Alcoholic and lactic acid fermentation.

In *Lactobacillus brevis* and *Leuconostoc* species, the EMP, HMP and ED pathways are absent, and these organisms possess the *phosphoketolase pathway*. The overall reaction is :

$$\text{Glucose} \longrightarrow \text{Lactic acid} + \text{Ethanol} + CO_2$$

Glucose is first metabolized to *pyruvic acid, acetic acid* and CO_2

$$\text{Glucose} \longrightarrow \text{Pyruvic acid} + \text{Acetic acid} + CO_2 + 6H$$

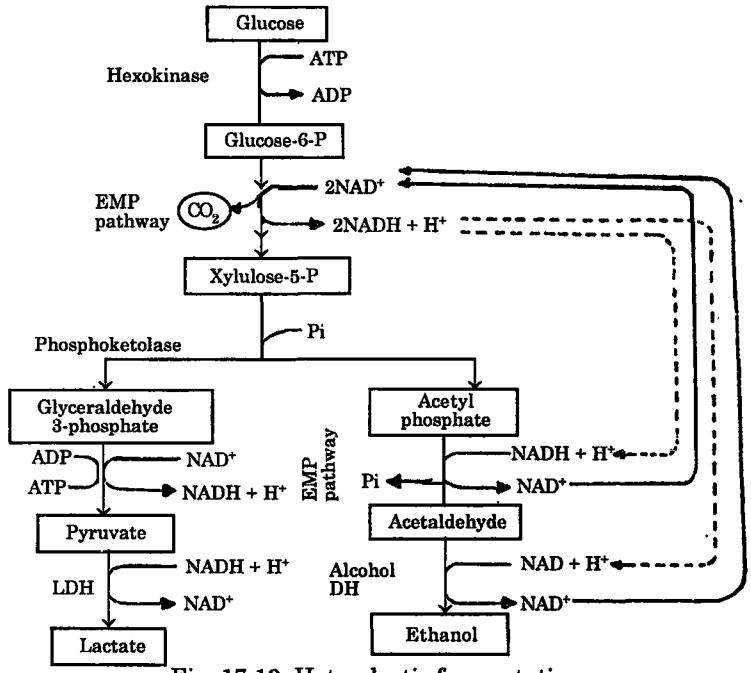


Fig. 17.19. Heterolactic fermentation.

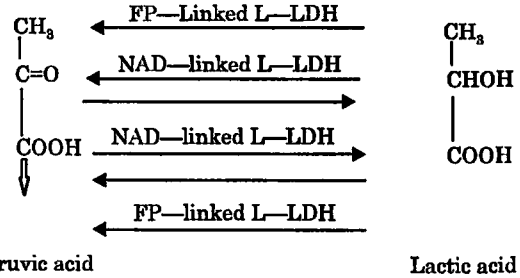
Pyruvic acid is then reduced to lactic acid, and acetic acid to ethanol through acetaldehyde.

$$\text{Glucose} \begin{cases} \text{Pyruvic acid} \xrightarrow{2H} \text{Lactic acid} \\ \text{Acetic acid} \xrightarrow{2H} \text{Acetaldehyde} \xrightarrow{2H} \text{Ethanol} \end{cases}$$

For every mole of glucose fermented there is a net yield of one mole of ATP. Thus heterolactic fermentation produces only half the energy produced by homolactic fermentation.

Optical Activity of Lactate

The optical activity of the lactate produced depends upon the stereospecificity of *lactate dehydrogenase* (LDH), and also on whether the organism possesses *lactate racemase*. Bacterial LDHs are of two types, *NAD-linked* and *flavine-linked*. Both types exist in the stereo-specific D and L forms. Flavine-linked dehydrogenases work only in one direction, from *lactate* to *pyruvate*, while NAD-linked dehydrogenases work in both directions.



In *muscle cells,* the product of lactic acid fermentation is *L-lactate.* In many *microorganisms* it is the *D-isomer* or a mixture of *D-and L-isomers.* An organism may contain *L*-LDH or *D*-LDH or both. Some microorganisms possess only one isomer, but possess *lactate racemase* which catalyses the interconversion of the two isomers (FIg. 17.20).

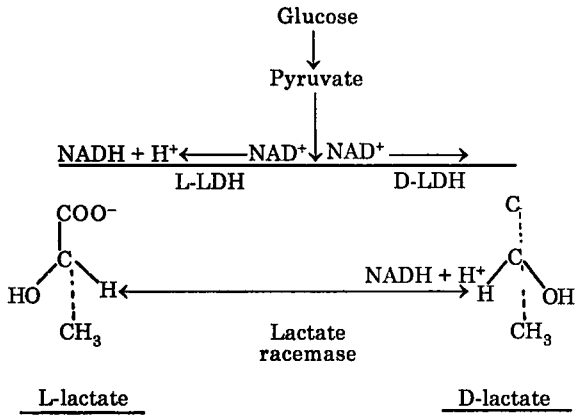


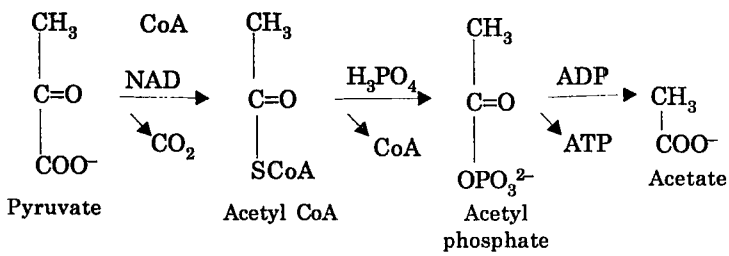
Fig. 17.20. Interconversion of L-lactate and D-lactate by lactate racemase.

III. Propionic Acid Fermentation (Fig. 17.21)

The propionic acid bacteria belong to the genus *Propionibacterium.* Those bacteria can ferment *glucose* or *lactate* to *propionic acid* under anaerobic conditions. Propionic acid bacteria are especially numerous in the digestive tract of ruminants. The cellulose digesting bacteria in the rumen can digest *cellulose to* form *glucose,* which is then converted to *lactate.* The propionic acid bacteria can then convert the glucose or lactate into *propionic* and *acetic acids,* along with some *succinic acid.* The propionic and acetic acids are absorbed into the blood stream of the host.

1. Propionic acid fermentation from glucose (1), (2) : Glucose forms pyruvate by the EMP pathways (1) and (2). The pathway is then splitted into two, one branch forming *acetate* and CO_2 and the other *propionate.*

(3) During *acetate formation,* pyruvate reacts with *lactate* and is converted to *acetyl CoA* by *pyruvate dehydrogenase.* This enzyme is different from the pyruvate dehydrogenase which forms acetyl CoA in the TAC cycle. (4) the acetyl CoA is prosphorylated to *acetyl phosphate,* the phosphate being supplied by H_3PO_4. (5) The phosphate group is then transferred to ADP to form ATP and *acetate.*



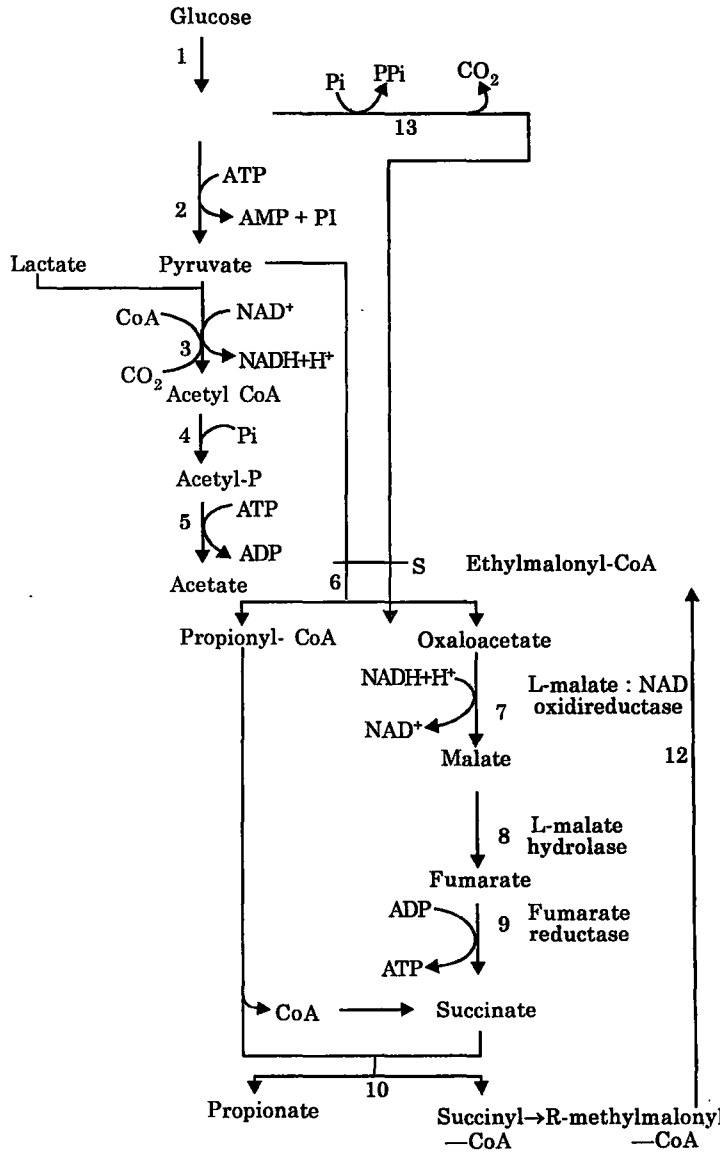


Fig. 17.21. Propionic acid fermentation.

The propionate branch can start either with *pyruvate* or *phosphoenol pyruvate.*

(6) *Pyruvate* reacts with 5 *methylmalonyl CoA* to form *propionyl CoA* and *oxaloacetate.*

Pyruvate + 5, methylmalonyl - CoA ⟶ Propionyl — CoA + Oxaloacetate

(7-9) Oxaloacetate gives rise to *malate, fumarate* and *succinate via* reverse TCA cycle reactions. (9) The conversion of *fumarate* to *succinate* takes place by *fumarate reductase,* which does not occur in the TCA cycle. (10) Propionyl-CoA transfers its coenzyme (CoA) to succinate, and *propionate* and *succinyl-CoA* are formed.

Succinate + Propionyl — CoA ⟶ Propionate + Succinyl — CoA

(11) Succinyl-CoA forms *R-methylmalonyl-CoA* under the catalytic action of the enzyme *methylmalonyl-CoA isomerase.* (12) *R methyl-malonyl-CoA* is converted into *S-methyl malonyl-*

CoA by the enzyme *methylmalonyl-CoA racemase.* (13) If succinate accumulates during fermentation of glucose, the cycle is broken. Oxaloacetate must therefore be generated by carbon dioxide fixation. This reaction is catalysed by *phosphoenolpyruvate carboxykinase.*

$$\text{Phosphoenolpyruvate} + CO_2 + \text{Pi} \longrightarrow \text{Oxaloacetate}$$

There is high yield of ATP in the propionic acid pathway. About 6 moles of ATP are formed per 1 - 5 moles of glucose in cells. Reactions (2) and (5) would together yield 4 moles of ATP. The other two moles must therefore arise from reaction (9), where electron-transport coupled phosphorylation may occur. When reaction (13) is operating the yield of ATP would decrease, since less phosphoenol pyruvate would be available for conversion to pyruvate.

2. Propionic acid fermentation from lactate : In *Clostridium propionicum* and *Bacteriodes ruminocola* there is an entirely different mechanism for production of propionic acid. The route from lactate is :

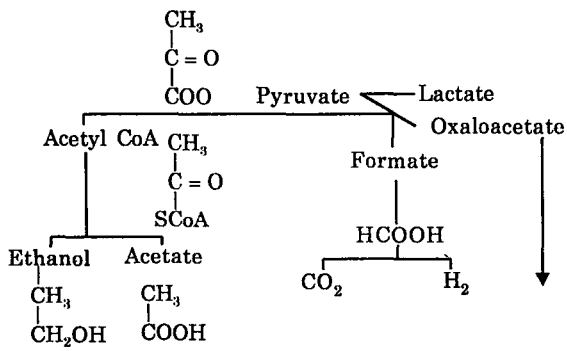
$$\text{Lactate} \longrightarrow \text{Acrylate} \longrightarrow \text{Propionate}$$

It has been suggested that the conversion of lactate propionate takes place via the CoA esters of lactate, acrylate and propionate (*lactolyl*-CoA and *propionic* CoA, respectively). It is, however, not certain whether this pathway is applicable to C-*propionicum* and B-*ruminicola.*

IV. Formic Acid Fermentation

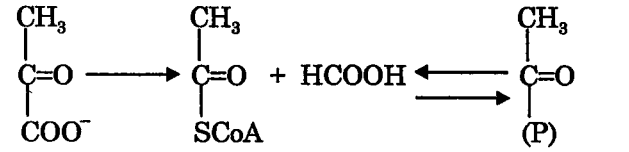
Formic acid fermentation is found in a number of bacteria, especially members of the Enterobacteriaceae. Metabolism of pyruvate produces several different products which vary in different organisms, but *formate* is always one of them. Bacteria carrying out formic acid fermentation can be divided into three groups : (1) *mixed acid* producers, (2) *butanediol* producers and (3) *butyric acid producers.*

1. Mixed acid producers : E-coli is the most characteristic member of this group. Glucose is converted into *ethanol* and *acetate* and either *formate* of its derived products CO_2 and H_2. Some glucose is also converted into *lactate* and *succinate.*



Glucose is first converted to *pyruvate* with the formation of 2ATP and 2NADH.

Pyruvate is cleaved by the pyruvate formate-lyase-reaction (phosphoroclastic reaction) to yield-*formate* and *acetyl*-CoA. Acetyl-CoA usually reacts further with phosphate to form *acetyl phosphate.* Acetyl CoA and acetyl phosphate are held in equilibrium by *phosphate acetyl-transferase.* Acetyl phosphate can transfer its phosphate group to ADP to form ATP.



(A) Formation of acetate : The first step in acetate formation is the breakdown of pyruvate to acetyl CoA. The reaction requires *pyruvate dehydrogenase,* lipoate and TPP. Three systems operate under anaerobic conditions.

(*a*) *Without formate formation* (characteristic of clostridia).

Pyruvate ⟶ Acetyl CoA/Acetyl phosphate + CO_2

(*b*) *With formation of formate* (characteristic of Enterobacteriaceae)

Pyruvate ⟶ Acetyl.CoA/Acetyl phosphate ⟶ Formate ⟶ $H_2 + CO_2$

(*c*) *Direct decarboxylation* (mainly in yeasts and higher plants)

Pyruvate ⟶ Acetaldehyde + CO_2

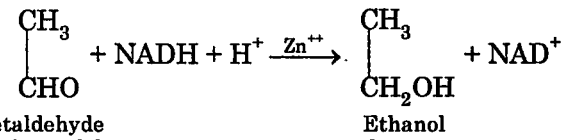
Two reactions now convert *acetyl CoA* to *acetate.*

Reaction 1 : Acetyl CoA + Orthophosphate ⟶ CoA + Acetyl phosphate

Reaction 2 : Acetyl phosphate + ADP ⟶ Acetate + ATP

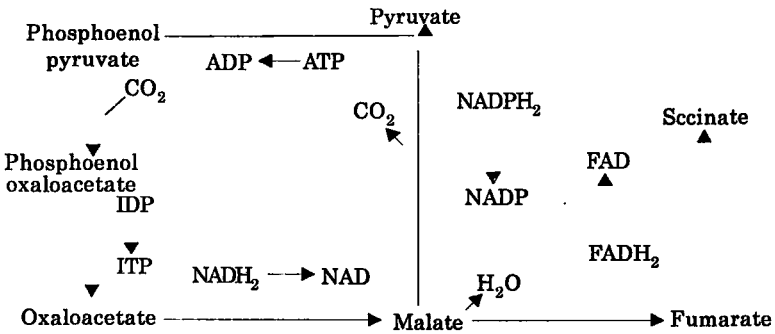
Reaction 1 is catalysed by *phosphate acetyltransferase* and reaction 2 by *acetate kinase.* One mole of ATP is generated for each mole of acetate formed.

(B) Formation of ethanol : Half of the *acetyl*-CoA is cleaved to *acetate,* while the other half is reduced to *ethanol* in two steps. In the first step *acetaldehyde* is produced, while in the second step acetaldehyde is reduced by the NADH produced during the initial oxidation of triose phosphate to yield *ethanol.* The formation of ethanol from acetaldehyde requires *alcohol dehydrogenase.*



(C) Formation of formate : Some Enterobacteria convert glucose into formate which may accumulate (*e.g., Shigella*). The conversion of formate to CO_2 and H_2 takes place under acidic conditions, and is catalysed by *formic hydrogen-lyase,* which consists of two enzymes, *formate dehydrogenase* and *membrane bound hydrogenase.*

(D) Formation of succinate : Some glucose is converted into *succinate.* The Enterobacteria can form *oxaloacetate* and *succinate* from pyruvate by a reversed TCA cycle.



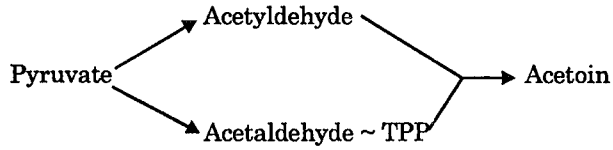
(E) Formation of lactate : Some of the saccharolytic clostridia possess *lactate dehydrogenase* and are able to reduce pyruvate to *lactic acid* if grown under iron-deficient conditions.

2. Butanediol producers : In this group are included such genera as *Aerobacter, Serratia* and *Bacillus* spp. Some of pyruvate is metabolized, as in mixed acid fermentation, but most of it is condensed with decarboxylation to yield α-*acetolactate.* This in turn is decarboxylated to *acetoin* (acetylmethyl-carbinol), which is reduced with $NADH_2$ to *2, 3-butanediol.*

$$\text{2 Pyruvate} \xrightarrow{TTP, Mg^{++}} \underset{+CO_2}{\alpha\text{-Acetolactate}} \longrightarrow \underset{+CO_2}{\text{Acetoin}} \xrightarrow{NADH_2} \underset{+NAD}{\text{Butanediol}}$$

Formation of acetoin : Acetoin can be formed from *acetaldehyde, acetolactate* and from *diacetyl.* The postulated schemes are as follows :

(*i*) In yeasts there is direct *formation of acetoin from pyruvate*



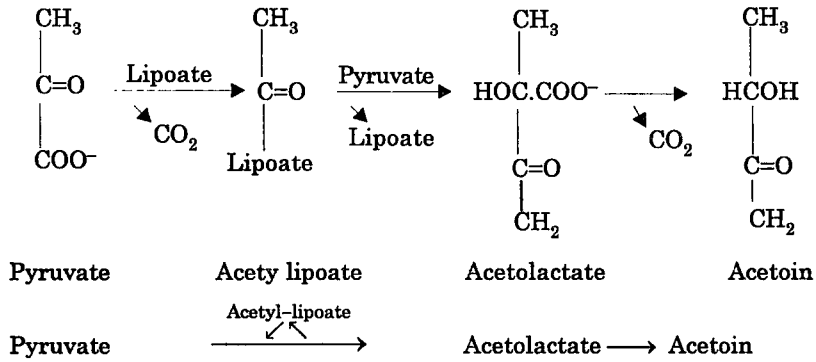
(*ii*) Formation of acetolactate from *pyruvate takes* place by pyruvate decarboxylation and aldehyde transfer. *Pyruvate* first reacts with *lipoate* to form *acetyl lipoate* ('activated acetate') and CO_2 is released.

$$\text{Pyruvate} + \text{Lipoate} \longrightarrow \text{Acetyl lipoate}$$

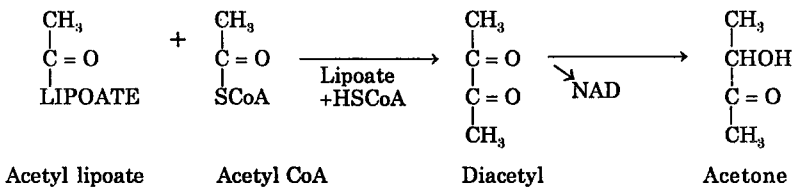
Acetyl lipoate then combines with a second molecule of *pyruvate* to produce *acetolactate,* and lipoate is released.

$$\text{Acetyl lipoate} + \text{Pyruvate} \longrightarrow \text{Acetolactate} + \text{Lipoate}$$

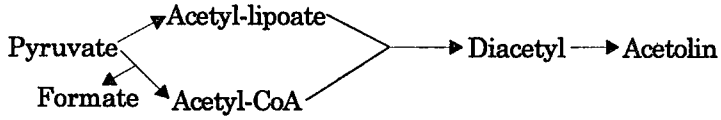
Acetolactate is then decarboxylated by *aceto-lactate decarboxylase* to form *acetoin.*



(*iii*) Formation of acetoin from *diacetyl* takes place in organisms which can simultaneously form *acetyl-CoA* by the phosphoroclastic split, and *acetyl-lipoate* with *pyruvate dehydrogenase.* Both 'active acetates' are then condensed to form *diacetyl.* Diacetyl is reduced to *acetoin* by *acetoin dehydrogenase.*



The scheme may be summarised thus :



Formation of butanediol : A number of bacteria are able to produce *2, 3, butanediol* in addition to mixed acid products.

3. Butyric acid producers (Figs. 17.22 and 17.23) : Butyric acid fermentation is carried out by saccharolytic clostridia. Several products may be formed, but butyric acid is always one of them, either in the intermediate or terminal stage. Different species of *Clostridium* produce different products.

Species	*Products*
Cl. butyricum	Acetate, butyrate, CO_2, H_2
Cl. acetobutylicum	Acetone and butanol + above
Cl. butylicum	Isopropanol from acetone.

Clostridia have a cyclic mechanism which results in the formation of *butyric acid,* a considerably lesser acid end product than acetate. The formation of butyric acid from acetyl CoA by clostridia is as follows :

1. Two *acetyl* CoA molecules condense to form *acetoacetyl* CoA, the catalysing enzyme being *acetyl transferase.*
2. Acetoacetyl-CoA is reduced to β-*hydroxybutyryl*-CoA, and $NADH_2$ is oxidised to NAD. The catalysing enzyme is 3-*hydroxy butyrate dehydrogenase.*
3. β-Hydroxybutyryl-CoA is reduced by *enoyl*-CoA *hydratase* to form *crotonyl-CoA* and water.
4. Crotonyl-CoA is further reduced to *butyryl* CoA by an NAD *linked dehydrogenase.*
5. Butyrl CoA and acetate act together with a fatty acid CoA-*transferase* to form *acetyl-CoA* and *butyrate.* The acetyl CoA can now re-enter the reaction cycle.

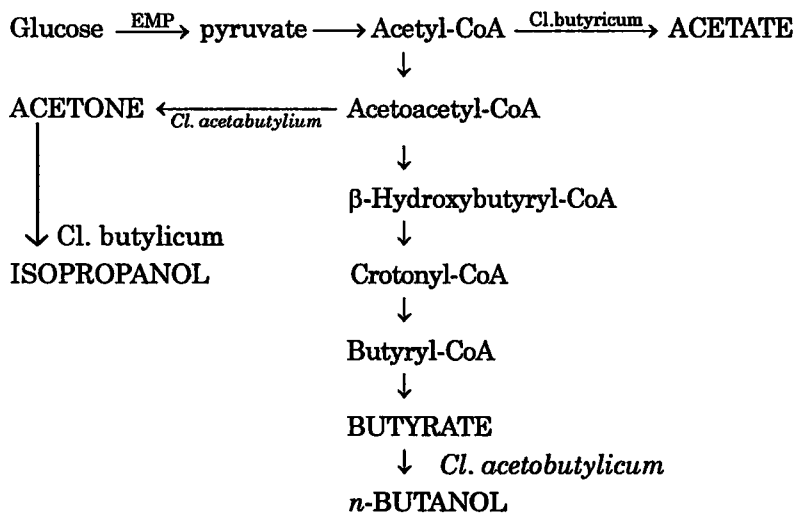


Fig. 17.22. Pathway in the fermentation of glucose by clostridia.

Interrelationship of the EMP, HMP and ED Pathways

(Figs. 17.24, 17.25 and 17.26)

The EMP pathway is present in all organisms that degrade glucose. The enzymes of the pathway are located in the cytosol, and the pathway therefore does not require any structural organisation for operation. This indicates that the pathway arose early in evolutionary history, before the development of intracellular structure.

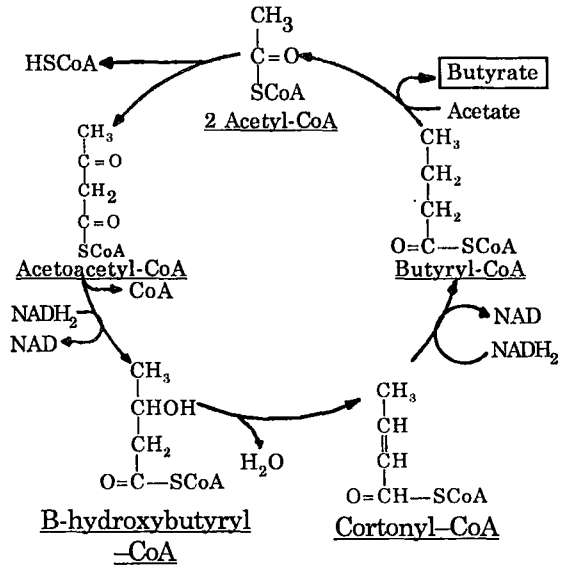


Fig. 17.23. Formation of butyrate from acetyl-CoA by clostridia.

The EMP pathway occurs in prokaryotes as well as in higher plants and animals. It is identical in all organisms up to the point of pyruvate formation. Differences begin from this state. In most living forms the end products of anaerobic decomposition of glucose are *lactic acid* and *ethyl alcohol*. Various photosynthetic anaerobes however, use organic compounds as terminal electron acceptors. In most animals and aerobic microorganisms, pyruvic acid is metabolised to *acetyl*, CoA, which then enters the *Krebs cycle*.

The intermediates of the EMP pathway serve as starting materials for cellular biosynthesis (Fig. 17.26). Instead of being oxidized further, they are shunted into biosynthetic pathways for the sysnthesis of particular cellular compounds.

The Krebs cycle no longer functions as a cycle under anaerobic conditions when there is no terminal respiration of acetate. It forms a branched biosynthetic pathway from oxaloacetate. One branch is *reductive*, and is the reversal of the usual pathway. It leads to the formation of *succinate*. The other branch is the usual *oxidative* pathway to *α-ketoglutarate*.

The splitting of the cycle into a reversed reductive branch and a normal oxidative branch enables the biosynthesis of succinate and α-ketoglutarate, which are essential biosynthesis intermediates.

The HMP pathway appears to supplement the EMP pathway in the majority of cell types. In *Saccharomyces cerevisiae*, which metabolizes almost all glucose through the EMP pathway, as much as 30% of the glucose is metabolized by the HMP pathway under anaerobic conditions. The HMP pathway is believed to have developed initially in anaerobic prokaryotes, and is found in most present day organisms. The main functions of the pathway appear to be (*i*) to supply ribose for nucleotide synthesis and (*ii*) to supply reduced coenzymes for reductive biosynthesis. The HMP pathway is also called the *pentose phosphate shunt*, because certain reactions of the EMP pathway are bypassed in this scheme. The HMP pathway utilizes NADP instead of NAD as an electron donor. The net result of the pathway is the oxidation of glucose to glyceraldehyde-3-phosphate.

The heterofermentative lactic acid bacteria metabolize glucose to pyruvic acid and acetic acid acid through the HMP pathways. A molecule of glucose yields only one molecule of pyruvic acid. One molecule of ATP is used and two are generated, resulting in a net gain of one ATP molecule. Pyruvic acid is reduced to *lactic acid*, and acetic acid to *accetaldehyde* and finally *ethanol*.

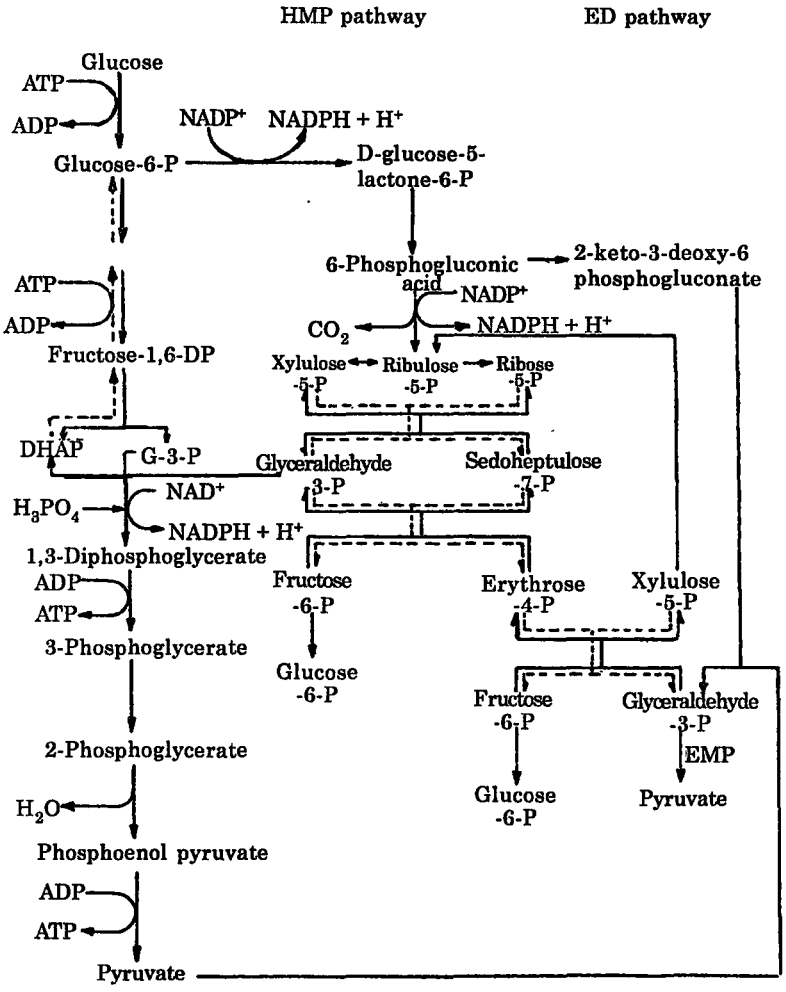


Fig. 17.24. Interrelationship between the EMP, HMP and ED pathways.

The ED pathway, like the HMP pathway, produces only one molecule of ATP per molecule of glucose. It is found only in a few microorganisms, mostly bacteria of the genus *pseudomonas*.

Oxidation and Reduction

In the 18th century Lavoisier showed that combination of inorganic materials and metabolism of living organisms were fundamentally the same processes. Both are processes of *oxidation,* in which oxygen is consumed and carbon dioxide and water produced. The definition of oxidation has since then undergone a chnage. In living systems *oxidation* is the *removal* of hydrogen atoms from a molecule, and *reduction* is the *addition* of hydrogen atoms from a molecule, and *reduction* is the *addition* of hydrogen atoms. Removal of a hydrogen atom in affect means the removal of a proton and an electron. Both oxidation and reduction go on simultaneously. When one substance *releasing* hydrogen (*hydrogen donor*) is said to be *oxidized,* while the substance *accepting* hydrogen (*hydrogen acceptor*) is said to be *reduced.* The release of energy from a cell may be entirely independent of oxygen, as in anaerobic organims.

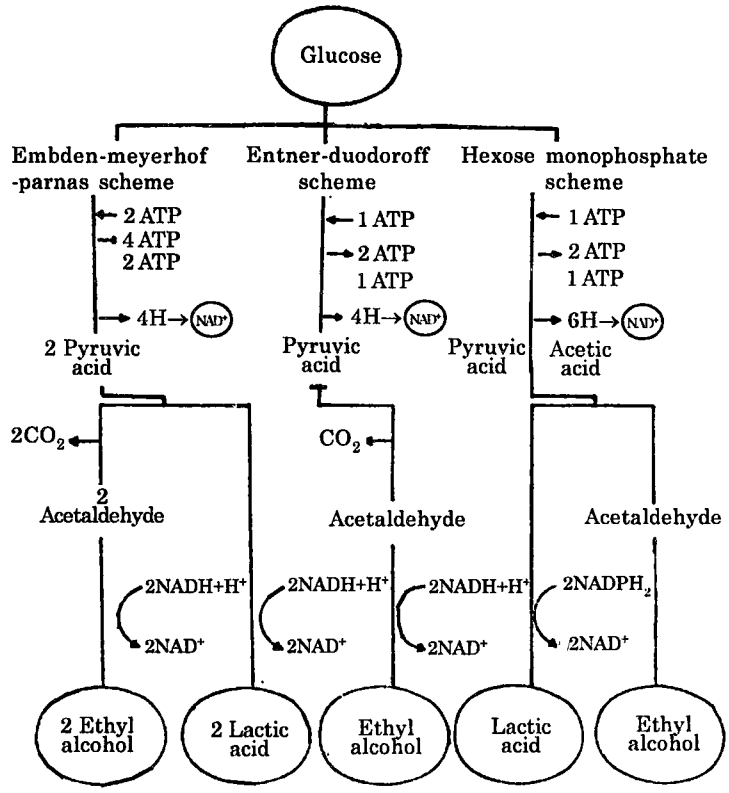


Fig. 17.25. The fate of pyruvic acid in some of the pathways of glucose metabolism.

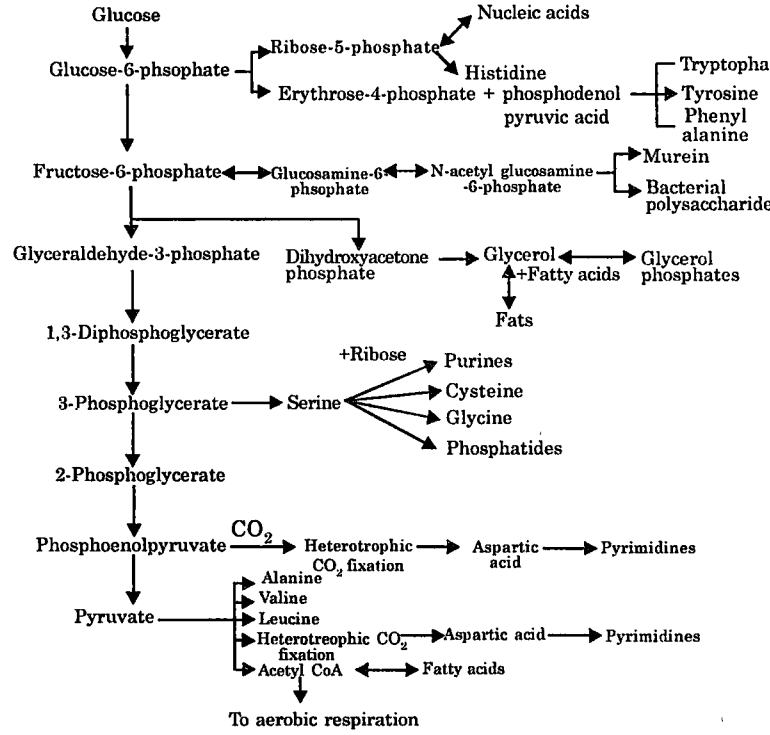


Fig. 17.26. Relationship between the EMP pathway and other metabolic pathways.

Electron Transport System or Respiratory Chain

The hydrogen and electron transport system consists of several hydrogen and electron acceptors. Biological oxidation-reduction reactions involve both hydrogen and electron acceptors. Hydrogen or electrons are passed from one acceptor to another. There are several *intermediate* hydrogen acceptors, and a *final* hydrogen acceptor which is molecular oxygen.

At various stages in glycolysis, oxidation of pyruvate and the Krebs citric acid cycle, removal of hydrogens (2H) takes place. The initial acceptor of hydrogens released from the substrates is NAD, except in one case. The exception is the hydrogens released from succinate, which are accepted by FAD of flavoprotein.

The respiratory chain is located in the inner membrane of the mitochondrion. On chemical treatment it breaks down into five *complexes* (I-V) and two mobile carriers.

Complex I consists of NADH dehydrogenase (of which FMN is the prosthetic group) and 6 *non-haeme iron (NHI) proteins* with *iron sulphur* (Fe-S) centres.

Complex II consists of *succinate dehydrogenase,* which contains covalently bound FAD as the prosthetic group, two Fe-S centres, and another Fe-S protein. Cytochrome *b* (absorbance 557.5 nm) has also been reported in complex II.

Between complexes II and III is the mobile carrier *coenzyme* Q (CoQ) or *ubiquinone* (UQ).

Complex III contains cytochromes b_K, b_T and c_1 and a non-haeme iron protein with an Fe-S centres.

Between complexes III and IV is the mobile carrier *cytochrome c.*

Complex IV (Cytochrome oxidase) contains cytochromes *a.* a_3 and two copper centres (Cuα and Cuβ).

Complex V (ATPase complex), consists of a *headpiece* (F_1, ATPase proper), a *stalk* of F_5 (OSCP) and F_6 and a *basepiece* (Fo) containing the protein channel.

COMPONENTS OF THE RESPIRATORY CHAIN

1. Substrate Dehydrogenases (Nicotinamide Nucleotide Dehydrogenases)

Biological oxidations are catalysed by *enzymes* which function in conjunction with *coenzymes* or electron carriers. The *nicotinamide nucleotides* are *coenzymes* for dehydrogenases (enzymes). They are also referred to as the *pyridine nucleotides,* because nicotinamide is a *substituted pyridine.*

The substrate dehydrogenases contain either *nicotinamide adenine dinucleotide* (NAD) or *nicotinamide adenine dinucleotide phosphate* (NADP), a phosphorylated form of NAD (Figs. 17.27 and 17.28). The old names for NAD were *disphosphopyridine nucleotide* (DPN) or *coenzyme I,* and that for NADP were *triphosphopyridine nucleotide* (TPN) or *coenzyme II.* NAD and NADP act as hydrogen carriers through alternate reduction and oxidation of the pyridine ring. The reduced coenzymes are NADH and NADPH, respectively. In general, NAD serves as an *oxidizing agent* by accepting hydrogen from substrates. NADP on the other hand is more often used as a *reducing agent* in biosynthetic processes, and is reduced to NADPH. NAD and NADP act as coenzymes. They are essential for enzyme activity, but easily dissociate from the apoprotein. On doing so they lose their biological activity.

NAD consists of two nucleotides, *nicotinoamide ribose phosphate* and *adenine ribose phosphate* : hence the name nicotinamide adenine diphosphate. The reactive part of the NAD molecule is the *nicotinamide ring.* The N of nicotinamide is positively charged, and so NAD is also written as NAD^+.NAD is the major electron acceptor of dehydrogenation reactions in which a pair of hydrogens (2H) are released. The two hydrogens consist of two protons (H^+) and two

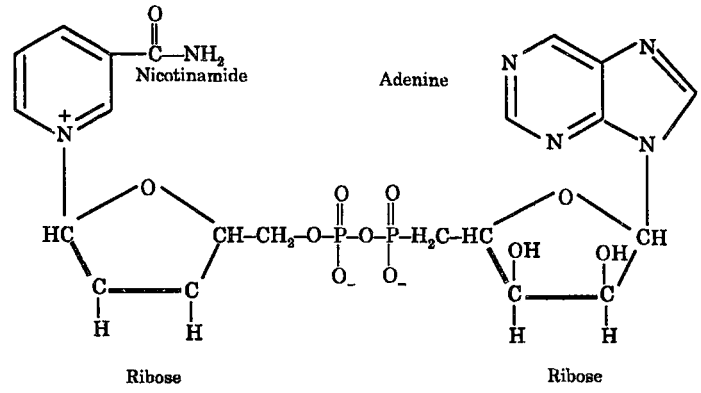


Fig. 17.27 (A). Nicotinamide adenine dinucleotide (NAD^+) or diphosphopyridine nucleotide (DPN^+)

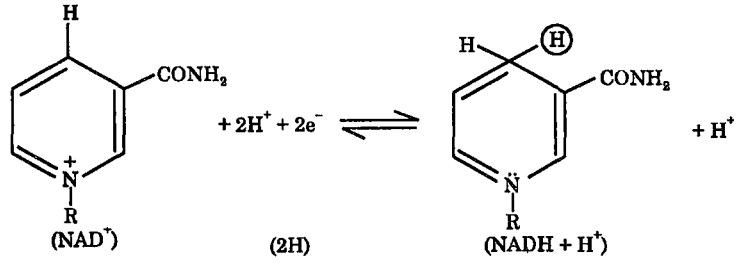


Fig. 17.27 (B). NAD^+ on accepting 2H becomes NADH + H^+. One hydrogen is accepted by NAD^+ and the other is released as a hydrogen ion (H^+).

electrons, (e^-). Since NAD^+ already has a positive charge, it accepts only one hydrogen proton (H^+) and two electrons, the equivalent of a *hydride ion.* The second proton is lost to the surrounding medium in which it is soluble. NAD^+ is reduced to NADH + H^+

$$NAD^+ + 2H \longrightarrow NADH + H^+$$

or $$NAD^+ + (2H^+ + 2e^-) \longrightarrow NADH + H^+$$

While writing, the H^+ is often omitted and the reduced form is written as NADH. Sometimes it is also written as $NADH_2$.

NADP has a phosphate group instead of the 2′-hydroxyl group of NAD. On accepting 2H it is reduced to NADPH + H^+.

$$NADP + 2H \longrightarrow NADPH + H^+$$

NADPH is the electron donor in most reductive biosyntheses, while NADH is mainly used for ATP generation.

2. Flavoproteins

Both complexes I and II contain dehydrogenases. Complex I contains *NADH dehydrogenase* while, complex II contains *succinic dehydrogenase.* These dehydrogenases contain *flavoproteins* as the main redox

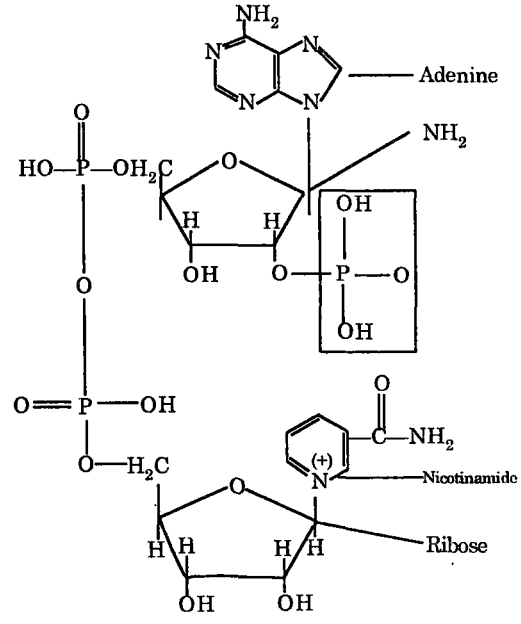


Fig. 17.28. Nicotinamide adenine dinucleotide phosphate ($NADP^+$). When a phosphate group is added to NAD^+ it becomes $NADP^+$.

component, and also one or more *iron sulphur* (Fe-S) *proteins*. The flavoprotein of complex I (FP_N) consists of an *apoprotein* plus tightly bound *flavin mononucleotide* (FMN) as its *prosthetic group*. The flavoprotein of succinic dehydrogenase (FP_s) contains *flavin adenine dinucleotide* (FAD) as the prosthetic group. Since both FMN and FAD are tightly bound to their apoproteins, they act as prosthetic groups rather than as coenzymes.

FAD consists of two halves, *flavin mononucleotide* (FMN) and *adenosine monophosphate* (AMP) (Fig. 17.29). The components of FAD are *flavin, D-ribitol, D-ribose, adenine* and two *phosphates*. A break between the two phosphates results in FMN and *adenine D-ribose phosphate* (AMP). *Flavin D-ribitol* contains a three-ringed *isoalloxazine* nucleus which is the reactive part. In contrast to NAD^+, this nucleus can accept both hydrogens ($2H = 2H^+ + 2e^-$) released by the substrate molecule. FAD on accepting the hydrogens is reduced to $FADH_2$.

$$FAD + 2H \longrightarrow FADH_2$$

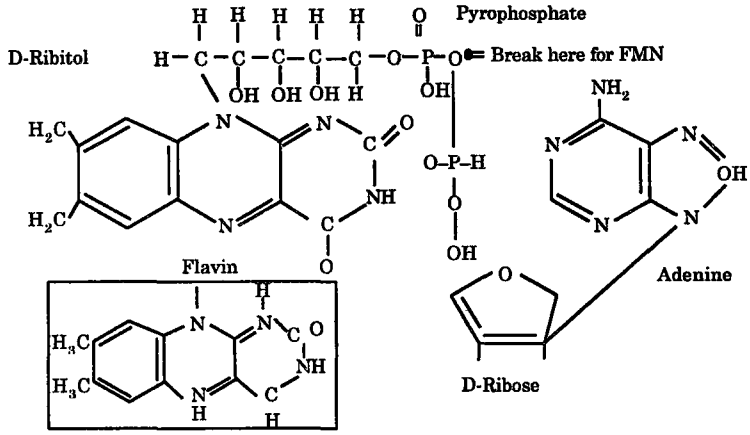


Fig. 17.29. Flavin adenine dinucleotide (FAD). FAD consists of two halves, flavin mononucleotide (FMN), shown on the left and adenosine monophosphate shown on the right, *inset* FMN on accepting (2H) is reduced to $FMNH_2$.

However, it is possible that *in vivo* the nucleus carries only one reducing equivalent. It would thus oscillate in the *semiquinone form* between the oxidized and the reduced forms.

FMN on accepting 2H from $NADH + H^+$ is reduced to $FMNH_2$.

$$NADH + H^+ + FMN \longrightarrow FMNH_2 + NAD^+$$

The standard redox potential of the $FADH_2$/FAD couple is –210 mV and that of the $FMNH_2$/FMN couple –190 mV. These values are, however, different when the flavins are bound to the apoprotein.

NADH dehydrogenase : This enzyme is also called *NADH; CoQ reductase* and is located in complex I. It consists of *flavoprotein*, which has *flavin mononucleotide* (FMN) as its 'prosthetic group', and *iron-sulphur* (Fe-S) centres. The molecular weight of the enzyme is about 550,000.

Succinic dehydrogenase (MW 70,000) is found in complex II. It contains covalently bound FAD as the prosthetic group and two Fe-S centres (Fe-S S1 and Fe-S S2). The FAD is covalently bound to the apoprotein though a *histidine* (His) residue. This covalent linkage permits raising of the standard redox potential of $FADH_2$/FAD from that of the free form (–210 mV) to that of succinate/fumarate (+ 30 mV). An additional Fe-S protein (MW 27,000) designated as Fe-S S3 is associated with succinic dehydrogenase to form complex II (succinate-CoQ reductase).

3. Iron Sulphur Proteins (Fe-S)

Some enzymes use metallic ions linked to the apoenzyme for transfer of electrons in oxidation-reduction reactions. Such enzymes are called *metalloproteins.* The commonly involved ions are those of *iron* and *copper.* Proteins that bind iron atoms in a lattice of sulphur atoms are called *non-haeme iron proteins* (NHI) or *iron sulphur proteins* (Fe-S). (Proteins containing iron chelated to a *porphyrin* group are called *haeme proteins*). Complex I contains 6 iron-sulphur centres, Fe-S N1a, Fe-S N1b, Fe-S N2, Fe-S N_3, Fe-SN_4 and Fe-S N_5 the 'N' indicating that the centre is a part of NADH dehydrogenase. There is a large difference in the E^0 values of centres N1, N3, N4, N5 (≤ -263 mV) and N2 (–20 mV). This indicates that electron transfer from (N1, N3, N4, N5) ⟶ N2 releases sufficient energy to synthesize ATP).

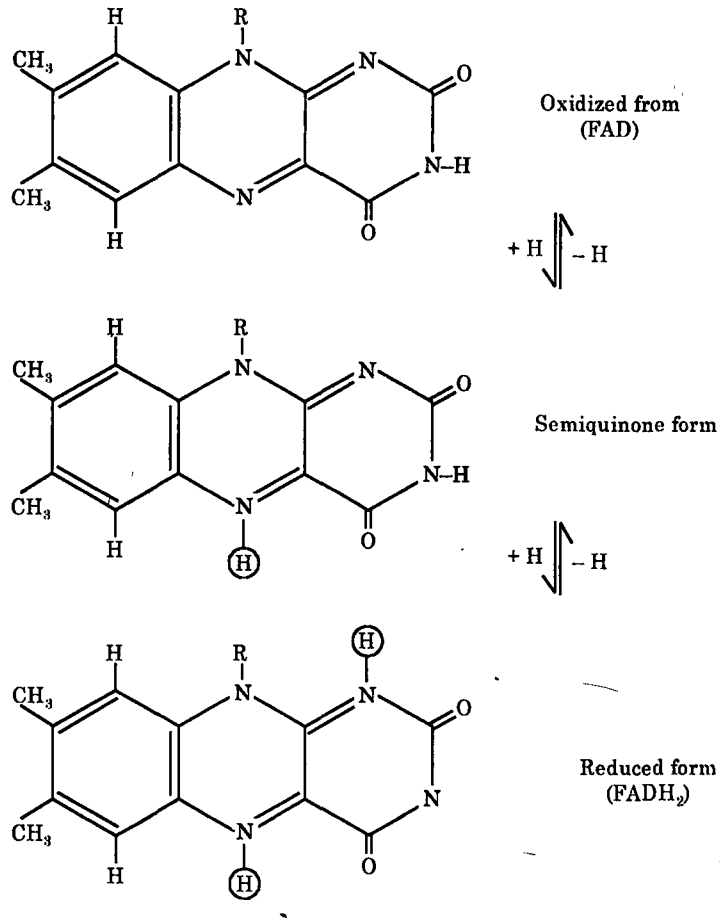


Fig. 17.30. Structure of the reactive, 3 ringed isoalloxazine nucleus of flavin adenine dinucleotide (FAD) during oxidized, semiquinone and reduced states.

The non-haeme iron in *succinate dehydrogenase* (complex II) is found in three centres, Fe-S S1, Fe-S S2 and Fe-S S3. The 'S' indicates that the centres are found in succinate dehydrogenase. Electron transfer probably takes place in the following direction FAD ⟶ Fe-S SI ⟶ FE-S S3. The $E^{0'}$ of Fe-S S2 is too low for it to be catalytically active. Complex III also contains a non-haeme iron protein (NHI) (MW 26,000) which has the iron-sulphur (F-S) centre.

4. Coenzyme Q (CoQ) or Ubiquinone (UQ)

Coenzyme Q is an electron carrier which if strictly speaking is not an enzyme. It is a lipid soluble quinone acting as a coenzyme. Structurally it has a *benzoquinone* nucleus attached to an unsaturated *isoprenoid* side chain (Fig. 17.31). The number of isoprenoid units varies from 6 to 10 (CoQ_{6-10}). Bacteria typically contain 6 isoprenoid units and this number is also found in some yeasts. Mammalian mitochondria contain 10 units. Coenzyme Q is able to diffuse rapidly in the mitochondrial membrane. This property is due to the isoprenoid tail which makes coenzyme Q highly nonpolar. Thus CoQ functions as a mobile electron carrier between the flavoproteins and the cytochromes.

Coenzyme Q is found in the mitochondria in the oxidized *quinone* form under aerobic conditions, and in the reduced *quinol* form under anaerobic conditions. The reduced form is called

hydroquinone ($CoQH_2$) or *dihydroxyubiquinone.* The partially reduced form is called *semiquinone.* It is relatively stable and can transfer one electron. Thus CoQ can take part in both one-electron transfer reactions (*semiquinone*) and two-electron transfers (*hydroquinone*).

Plastoquinone, which occurs in chloroplast and functions in photosynthesis, is closely related to coenzyme Q. It contains two CH_3 groups which replace the methoxyl ($-OCH_3$) groups of coenzyme Q. In some bacterial respiratory systems *menaquinone* takes the place of coenzyme Q. The polyisoprenoid side chain is also present in vitamins E and K.

5. The cytochromes

The 1925 Keilin gave the name 'cytochromes' to certain conjugated proteins. He identified three cytochromes which he named *a*, *b* and *c*. Since then about 50 cytochromes have been identified. They are named according to which of the original three they resemble, *e.g.*, q_1, a_3, c_1, c_2, c_3 etc. Three main types of cytochromes, the *b*, *c* and *a* types, have been distinguished in the mammalian respiratory chain. There are at least two *b* type cytochromes *cyt* b_K (after Keilin) and *cyt* b_T (transducing). The main *c*-type cytochromes are *cyt c* and *cyt* c_1. The main *a*-type cytochromes include *cyt a* a_3 (*cytochrome oxidase*).

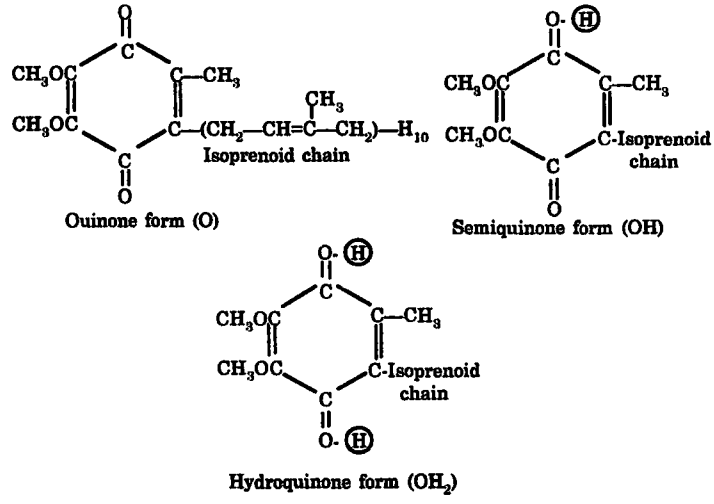
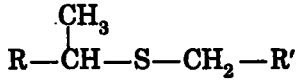


Fig. 17.31. Coenzyme Q (CoQ) or ubiquinone (UQ).

A. Quinone form (Q) Fully oxidized. Both oxygens connected to the ring by double bonds.
B. Semiquinone form (QH). Semi-oxidized. Hydrogen atom attached to one oxygen.
C. Hydroquinone form (QH_2). Fully reduced. Hydrogen atoms attached to both oxygens.

The cytochromes are conjugated proteins consisting of an *apoprotein* and a *prosthetic group* (*haeme*). The haeme consists of a *porphyrin* with a central *iron* atom. The porphyrins are complex carbon-nitrogen molecules that usually surround a metal ion (Me) to form *metalloporphyrins* (Fig. 17.32). The metal ion is magnesium in chlorophyll and iron in cytochromes and haemoglobin. Porphyrins are cyclic compounds consisting of 4 *pyrrole rings* linked by methylene bridges. The H substituted positions of the ring are named 1 to 8. The methylene bridges are called α, β, γ, and δ.

Haeme (iron protoporphyrin IX) is the prosthetic group of cytochromes *b*, c_1, *c*, myoglobin and haemoglobin (Table 17.3). In cytochromes *c* and c_1, the haeme is covalently attached to the protein by *thioether linkages.*



In cytochrome *b* the haeme is not covalently bonded to protein. The iron-poophyrin prosthetic group of cytochromes *a* and a_3 is called *haeme A.* Haeme A has a formyl group instead of one of the methyl groups of haeme in cytochromes *c* and c_1 and the vinyl group is replaced by a hydrocarbon group (long hydrophobic isoprenoid chain).

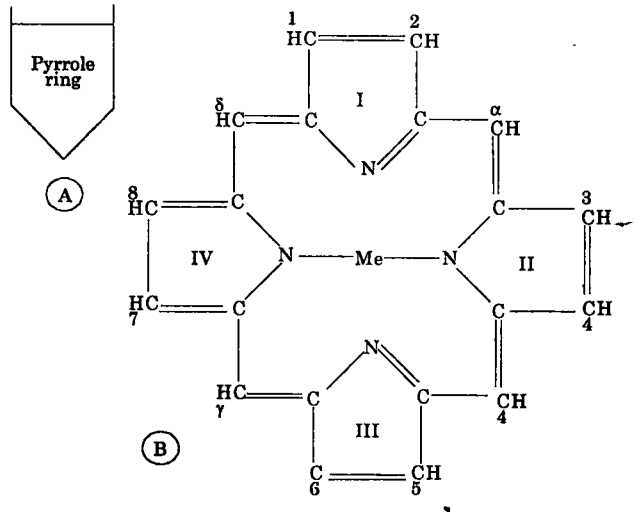


Fig. 17.32. General structure of porphyrin.

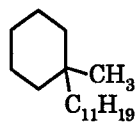
The substituent groups on carbons 2, 4, 5 and 8 of haeme differ in the different cytochromes.

Ionic iron exists in two forms, Fe^{2+} (ferrous) and Fe^{3+} (ferric). One form can be coverted into the other by loss or gain of an electron.

$$\text{(Ferric) } Fe^{3+} \underset{\text{Oxidation}}{\overset{\text{Reduction}}{\underset{-e}{\overset{+e}{\rightleftharpoons}}}} Fe^{2+} \text{ (Ferrous)}$$

Table 17.3

STRUCTURE OF HAEME

Haeme	*Carbon-2*	*Carbon-4*	*Carbon-5*	*Carbon-8*
a		$-CH=CH_2$	—	$-CHO$
b (protohaeme)	$-CH=CH_2$	$-CH=CH_2$	$-CH_3$	$-CH_3$
c (mesohaeme)	$-CH(CH_3)-$ S–Protein	$-CH(CH_3)-$ S–Protein	$-CH_3$	$-CH_3$

The Fe in the cytochrome molecule acts as an electron donor or acceptor. After *accepting* electrons the atom is *reduced,* while after *donating* electrons it is *oxidized.*

Cytochromes b : Complex III contains at least two *b*-type cytochromes, cytochrome b_K (*cyt* b_{560}) and *cytochrome* b_T (*cyt* b_{565}). The subscripts K and T stand for *Keilin-type* cytochrome *b* and *transducing* cytochrome *b*, respectively. The two cytochromes are either located in the middle of the membrane, or according to another view, they span the membrane. The $E^{0'}$ of *cyt* b_T is ATP variable. It changes from – 30 mV in the absence of ATP to + 245 mV in the presence of a high ATP concentration. It is therefore possible that *cyt* b_T plays a direct role in energy conservation.

The $E^{0'}$ cytochrome b_K does not vary with ATP and cyt b_K probably acts as a simple redox carrier. The *b* cytochrome contains *protohaeme* which is also found in bacterial cytochrome *c*.

Cytochrome c (Fig. 17.33) : The cytochromes *c* include the eukaryote mitochondrial *cyt c*, the bacterial photosynthetic *cyt* c_2 and several others of prokaryotic orign. They are not enzymes in the conventional sense but are cofactors for the proteins with which they interact. The single haeme prosthetic group is bound to the polypeptide chain of 82 to 134 residues near the amino terminus.

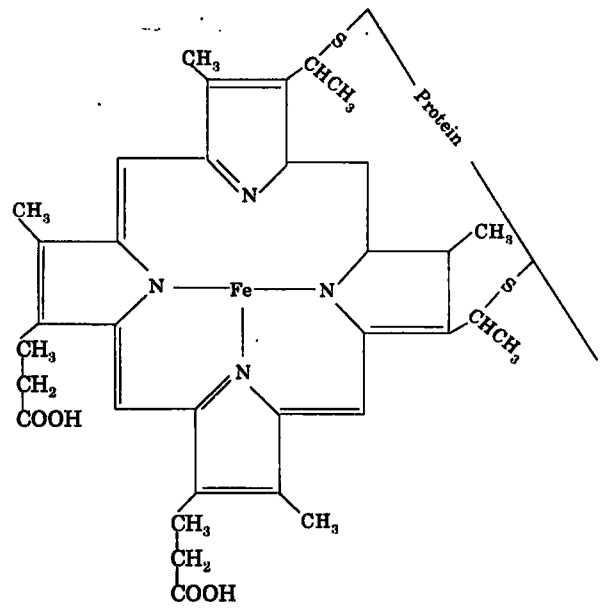


Fig. 17.33. Cytochrome *c*.

The molecular weight of cytochrome *c* is about 12,600 (104 amino acids). The standard redox potential (E^0) is + 254 mV for isolated *cyt c* and + 225 mV in mitochondria. The haeme group (*haeme c, mesohaeme*) is covalently bound to the polypeptide chain via thioether linkages from C-2 and C-4. There is also coordinate linkage between haeme and His-18 and Met-80 of the polypeptide chain. The polypeptide chain is wound around the haeme backbone.

The haeme is inserted into a hydrophobic slit within the protein in such a way that only parts of pyrrole rings I and IV are exposed. The amino acid sequences of the polypeptide chain of over 50 species has been determined. There are 28 invariant positions in the peptide chain.

Cytochrome c_1 is an intrinsic protein with MW 30,6000 (from beef heart mitochondria). Unlike cyt *c* it appears to be firmly bound to the membrane. The haeme-containing part of *cytochrome* c_1 is located on the C side of the membrane from, where it can easily transfer electrons to cyt c.

Cytochromes a, a_3 *(cytochrome c oxidase) :* Although cytochromes *a* and a_3 are considered to be separate cytochromes, they have never been physically separated and apparently cannot function independently. The functional unit of mammalian cytochrome oxidase consists of two *haeme molecules* and *two copper atoms* (Cuα and Cuβ). Cytochrome *a* does not react with carbon monoxide, and is therefore, not inhibited by it. Cytochrome a_3 on the other hand reacts with CO and is inhibited by it. CO competes with oxygen for the binding site on the ferrous ion.

The cytochrome oxidase complex is oriented perpendicularly to the plane of the membrane. It is about 83 A long, and therefore extends considerably beyond the 40-50 A lipid bilayer. The complex spans the membrane, with cytochrome *a* on the C-side and cytochrome a_3 on the membrane side.

Beef heart cytochrome oxidase consists of 6 subunits, I (MW 40,000), II (MW 22,500), III (MW 15,000), IV (MW 11,200), V (MW 9,800) and VI (MW 7,000). Subunits II, V and VI face the C-side of the membrane, I and IV are in the middle and III on the M-side.

Cytochrome *c* oxidase contains four units (cyt *a*, Cyt a_3 Cuα and Cuβ) which carry one electron each. Thus four electrons are stored in the enzyme prior to discharge. The four electrons ($4e^-$) combine with four protons ($4H^+$) and one molecule of oxygen to form water.

$$4H^+ + 4e^- + O_2 \longrightarrow 2H_2O$$

Table 17.4

STANDARD OXIDATION-REDUCTION POTENTIALS OF THE COMPONENTS OF THE RESPIRATORY CHAIN

Components	*From C.W. Jones (1976)*	*From Wilson et al. (1972, 76)*
Lactate/Pyruvate		– 0.185 V
Succinate/Fumarate	+ 30mV	+ 0.031 V
NADH/NAD^+	– 320 mV	– 0.320 V
NADPH/NADP	– 324 mV	
$FMNH_2$/FMN	– 190 mV (free)	
$FADH_2$/FAD	– 210 mV	
	+ 33 mV	~ – 0.045 V
	(+ apoprotein)	
Fe-S N1		
Fe-S N3	≥ – 245 mV	
Fe-5 N4		
Fe-S N2	– 20 mV	
Fe-S S1	+ 15 mV	
Fe-S S2	– 260 mV	
Fe-S S3	+ 60 mV	
CoQ or UQ	R–0mV	+ 0.10 V (isolated)
		+ 0.045 V
Cyt b_T (*b* 565)	– 20 mV	– 0.030 V
Cyt b_T + ATP	+ 240 mV	+ 0.245 C
Cyt b_K (*b* 560)	+ 65 mV	+ 0.030 V
Fe-S	.....	+ 0.028 V
Cyt C_1	+ 220	+ 0.215 V
Cyt *c*	+ 254 mV	+ 0.254 V (isolated)
		+ 0.215 V
Cyt *a*	+ 230 mV	+ 0.29 V (isolated)
		+ 0.210 V
Cyt a_3	+ 375 mV	+ 8.385 V
		+ 0.155 V (+ ATP)
Cuα and Cuβ	+ 280 mV	+ 0.245 V
H_2O/1/2 O_2	+ 820 mV	+ 0.815 V

The figures represent mid-point potentials ($E^{0'}$). The $E^{0'}$ value of cyt b_T changes from –0.030 V in the absence of ATP to + 0.245 V in presence of high ATP concentrations. However, $E^{0'}$ for cyt *a* drops from + 0.385 V to 0.155 V in the presence of ATP.

The redox potential (Table 21.4). The tendency for release and acceptance of electrons is called the *redox potential* ($E^{0'}$). The term 'redox' has been derived from reduction-oxidation. The redox potential is the *ratio of the reduced form to the oxidized form,* and is expressed in volts or millivolts. The redox potentials of various compounds of the hydrogen transport system at pH 7 are given in Table 17.4.

Transfer of hydrogen and electrons : As mentioned previously, hydrogen atoms are transferred from the substance to be oxidized to NAD^+. From NAD^+ the hydrogen atoms are transferred to FMN and CoQ. The hydrogen atom undergoes ionization, *i.e.*, it is split into an *electron* and a *proton*. In further stages there is no longer a transfer of hydrogens but of electrons. The electrons pass from coenzyme Q to cytochromes b_K, b_T, c_1, c, a and a_3. The protons are released free. The sequence of compounds in the hydrogen transport system is as follows :

$$NAD \longrightarrow FMN \longrightarrow Fe{-}S \longrightarrow CoQ \longrightarrow C_ytb_K \longrightarrow C_ytb_T \longrightarrow C_ytc_1 \longrightarrow Cytc \longrightarrow C_yt\ a.a_3$$

As the hydrogen atoms or electrons pass down the chain there is simultaneous oxidation of one coenzyme and a reduction of another at each step. The compounds of the electron transfer system first act as *oxidizing* agents by *accepting* electrons/hydrogens and then as *reducing agents* by *donating them*. Each substance in the respiratory chain is alternately oxidized and reduced. In the case of $NADH_2$ and $FMNH_2$ oxidation takes place by loss *of hydrogen*. For coenzyme Q and the cytochromes b_K, b_T, c_1, c, a and a_3, oxidation takes place by loss of electrons. At a time two electrons are released. Cytochromes b_K, b_T, c_1, c, a and a_3 *can accept only one electron at a time*. Therefore, the electrons are captured by *two* molecules each of the enzymes of the cytochrome system.

After transfer from cytochrome a_3, an electron and a proton are brought together. They combine to produce hydrogen. The final hydrogen acceptor is molecular oxygen, and H_2O is the final product. *It is because oxygen is used as the final acceptor in the hydrogen transport system that most organisms require oxygen in order to survive.*

Sites of Oxidative Phosphorylation (ATP Generation)

In 1941 S. Ochoa obtained the first reliable measurement of the P/O ratio. This ratio is the number of moles of ATP generated per atom of oxygen utilized in respiration, and is used as an index of oxidative phosphorylation. The P/O ratio is also equal to the number of moles of ATP formed per pair of electrons passing down the electron transfer system.

Lehininger (1951) showed that oxidation of NAD^+ linked substrates (*e.g.*, β-hydroxybutyrate) gave a P/O ratio of about 3. This indicated that there were 3 energy coupling sites between NADH and molecular oxygen. The aerobic oxidation of one mole of NADH through the mitochondrial respiratory chain gives rise to the synthesis of 3 moles of ATP from ADP and Pi. Complexes I, III, and IV each contains one energy coupling site which can interact with the ATP synthesizing system.

The P/O ratio of flavin linked substrates, *e.g., succinate,* was about 2. Thus, there are only two energy-coupling sites involved when succinate is the acceptor. The first energy coupling site (*i.e.*, comples I) is bypassed. This suggested that site I of oxidative phosphorylation was located between NADH and coenzyme Q.

Lehininger (1949) used tetramethyl phenylene diamine (TMPD) to introduce electrons at cytochrome *c*. Studies in which cytochrome *c* was directly used as an electron donor showed that only one ATP was generated, since sites I and II were bypassed. This indicated that site III was to the right of cytochrome *c*.

Slater (1955) showed that passage of electrons from succinate to cytochrome *c* gave a P/O ratio of only 1. This indicated that one ATP was generated at site II.

By such experiments it has been possible to localize the three energy coupling sites as follows :

Site I — between NADH and CoQ.

Site II — between cytochromes *b* and *c*.

Site III — between cytochrome *c and* O_2.

A general agreement about the stoichiometric relationships of the respiratory chain components is that for every two molecules of cytochrome *c* there is one of complex III, two of complex IV and 16 molecules of coenzyme Q (Slater, 1967). Inhibitor binding studies (Slater, 1972) indicate that there is only one ATP synthetase (complex V) for every complex III. Thus for every *three coupling sites* of each electron transfer chain there is only one ATP synthesizing and hydrolyzing unit.

Synthesis of ATP in the presence of oxygen is called *oxidative phosphorylation.* The normal site of oxidative phosphorylation in plant and animal cells is the mitochondrion. The term oxidative phosphorylation is used because ATP production requires oxygen in the last step of the series.

It is now possible to calculate the total number of ATP molecules produced when a molecule of glucose is metabolized through glycolysis and the Krebs cycle to produce carbon dioxide and water (Table 17.5 and 17.6).

Table 17.5

OUTLINE OF ATP PRODUCTION DURING AEROBIC OXIDATION OF GLUCOSE TO CO_2 AND H_2O

I. Glycolysis

(*i*) ATP produced directly .....4 molecules.

ATP used .....2 molecules.

Net gain .....2 molecules.

(*ii*) From hydrogens produced and sent down the hydrogen transport system (2 × 3) ..... 6 molecules.

II. Pyruvic Acid Oxidation

(*i*) From hydrogens produced and sent down the hydrogen transport system (2 × 3) ..... 6 molecules.

III. Krebs Cycle

(*i*) ATP produced directly .....2 molecules.

From hydrogen accepted by NAD (3 × 3) – 9 molecules.

Therefore for the two molecules of citrate metabolized (9 × 2) ..... 18 molecules.

(*iii*) From hydrogens accepted by FAD (1 × 2) – 2 molecules.

Therefore for the two molecule of citrate metabolized (2 × 2) ..... 4 molecules.

Total 38 molecules

Table 17.6

ATP PRODUCED DURING COMPLETE OXIDATION OF ONE MOLECULE OF GLUCOSE TO CARBON DIOXIDE AND WATER UNDER AEROBIC CONDITIONS

(I) GLYCOLYSIS		
(*i*) ATP produced by oxidation at substrate level		
Step 6.	1.3 – diposphoglycerate to 3-phosphoglycerate (1 × 2 = 2)	2 ATP
Step 9.	Phosphoenol pyruvate to pyruvate (1 × 2 = 2)	2 ATP
		4 ATP
ATP consumed		
Step 1.	Glucose to glucose-6-phosphate	1 ATP

Step 3.	Fructose 6-phosphate to fructose 1, 6-diphosphate	1 ATP
		2 ATP
	NET GAIN OF ATP (4 minus 2)	2 ATP
***(ii)* ATP from hydrogens produced and sent down the hydrogen transport system**		
Step 5.	PGAL to 1, 3-diphosphoglycerate (3 × 2 = 6)	6 ATP
TOTAL ATP PRODUCED DURING AEROBIC GLYCOLYSIS		8 ATP
(II) PYRUVATE OXIDATION		
ATP from hydrogens produced and sent down the hydrogen transport system,		
Pyruvate to acetyl CoA (3 × 2 = 6)		6 ATP
(III) KREBS CITRIC ACID CYCLE		
ATP produced by oxidation at substrate level		
Step 7. Succinyl CoA to succinate (1 × 2 = 2)		2 ATP
ATP from hydrogens produced and sent down the hydrogen transport system		
Step 4. Isocitrate to oxalosuccinate, NAD acceptor, (3 × 2 = 6)		6 ATP
Step 6. α-ketoglutarate to succinyl CoA, NAD acceptor, (3 × 2 = 6)		6 ATP
Step 8. Succinate to furmarate, FAD acceptor, (2 × 2 = 4)		4 ATP
Step 10. Malate to oxaloacetate, NAD acceptor, (3 × 2 = 6)		6 ATP
		24 ATP
TOTAL ATP PRODUCED		38 ATP

Table 17.7

SUBTANCES CONSUMED AND PRODUCED DURING DIFFERENT STAGES OF RESPIRATION

	Stage I Glycolysis	*Stage II Pyruvate Oxidation*	*Stage III Krebs Cycle*	*Stage IV Oxidative Phosphorylation*	*Total*
			Substances consume		
Glucose or products	Glucose	2 Pyruvates	2 Acetyl CoA	—	$C_6H_{12}O_6$
O_2	—	—	—	$6O_2$	$6O_2$
ADP	2 ADP	—	2 ADP	34 ADP	38 ADP
P	2P	—	2P	34 P	38 P
H	—	—	—	24H	—
CoA	—	2 CoA	—	—	—
H_2O	—	—	6 H_2O	—	—

	Substances produced				
Glucose or products	2 Pyruvate	2 Acetyl CoA	—	—	—
ATP	2 ATP	—	2 ATP	34 ATP	38 ATP
H	4H	4H	16 H	—	—
CO_2	—	2 CO_2	4 CO_2	—	6 CO_2
CoA	—	—	2 CoA	—	—
H_2	—	—	—	12 H_2O	6H_2O

Equations of the different stages of cell respiration.

(1) Stage I, Glycolysis :

$$\text{Glucose} + 2\,\text{ADP} + 2\text{P} \longrightarrow 2\,\text{Pyruvate} + 2\,\text{ATP} + 4\text{H}$$

(2) Stage II, Pyruvate Oxidation :

$$2\,\text{Pyruvate} + 2\,\text{CoA} \longrightarrow 2\,\text{Acetyl CoA} + 2\,CO_2 + 4\text{H}$$

(3) Stage III, Krebs Citric Acid Cycle :

$$2\,\text{Acetyl CoA} + 6H_2O + 2\,\text{ADP} + 2\text{P} \longrightarrow 4\,CO_2 + 2\,\text{ATP} + 16\,\text{H} + 2\,\text{CoA}$$

(4) Stage IV, Oxidative Phosphorylation :

$$24\,\text{H} + 6O_2 + 34\,\text{ADP} + 34\text{P} \longrightarrow 12\,H_2O + 34\,\text{ATP}$$

(5) Net Equation of Glucose Oxidation :

$$C_6H_{12}O_6 + 6O_2 + 38\,\text{ADP} + 38\text{P} \longrightarrow 6CO_2 + 6H_2O + 38\,\text{ATP}$$

Energy Relationships During Cell Respiration

One mole (180 gm) of glucose yields about 686,000 calories on complete combustion to carbon dioxide and water. The terminal phosphate group (~) of a mole of ATP contains about 10,000 calories. Therefore the 38 molecules of ATP represent an energy yield of 380,000 calories. This is 55% of the 686,000 calories originally present in glucose. Under aerobic conditions glycolysis (glucose to pyruvic acid) releases 50,000 calories, or 7% of the total 686,000 calories free enrgy change. The remaining 93% is released during oxidation of pyruvic acid to acetyl CoA, the Krebs cycle and oxidation by the hydrogen transport system (Fig. 17.34).

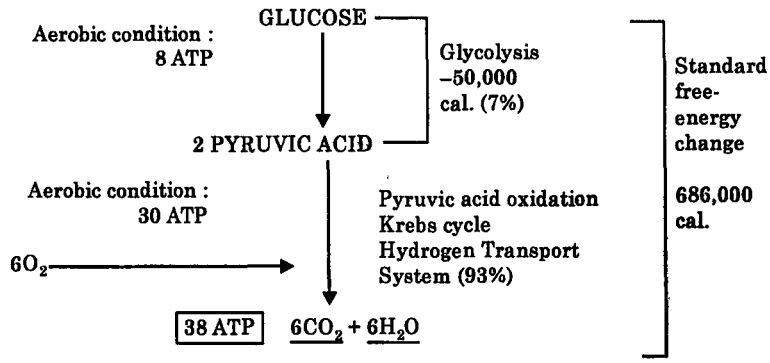


Fig. 17.34. Energy relationships during cell respiration.

Organism which do not have hydrogen transport systems do not require oxygen and are said to be *anaerobic*. Their mode of respiration is called *fermentation*, and results in various products

like ethanol, lactic acid and glycerol. During fermentation glycolysis may take place by the EMP pathway, HMP pathway, ED pathway, or other pathways. Organisms which have the hydrogen transport system require oxygen as the final hydrogen acceptor, and are said to be *aerobic*. In these organisms *aerobic glycolysis* takes place by the EMP pathway, and pyruvate acid enters the Krebs citric acid cycle.

The number of molecules of ATP produced differs in different types of respiration (Table 17.8). In anaerobic respiration the number of molecules of ATP formed is much less than in aerobic respiration.

Table 17.8

ATP PRODUCTION IN DIFFERENT TYPES OF RESPIRATION

Type of Respiration	*Organism or Tissue*	*Substrate and Products*	*ATP Generated*
Fermentation (Anaerobic)			
1. EMP scheme	Yeast	Glucose to ethanol	2 ATP
2. EMP scheme	Homofermentative lactic acid bacteria	Glucose to lactate	2 ATP
3. HMP scheme	Heterofermentative lactic acid bacteria	Glucose to lactate and ethanol	1 ATP
4. ED scheme	*Pseudomonas* (bacterium)	Glucose to ethanol	1 ATP
5. Alkaline fermentation	Yeast	Glucose to glycerol	No ATP
Aerobic respiration	Most animal cells		
1. Glycolysis, EMP scheme		Glucose to pyruvate	8 ATP
2. Pyruvic acid oxidation		Pyruvate to acetyl CoA (× 2)	6 ATP
3. Krebs cycle		Citrate to oxaloacetate,	24 ATP
		CO_2 and H_2O (× 2)	—
			38 APT
Muscle glycolysis			
1. EMP scheme anaerobic	Muscle	Glycogen to lactate	3 APT
Muscle respiration (aerobic)	Muscle		
1. Glycolysis (aerobic) EMP scheme		Glycogen to pyruvate	9 ATP
2. Pyruvic acid oxidation		Pyruvate to acetyl CoA (× 2)	6 ATP
3. Krebs cycle		Citrate to oxaloacetate	24 ATP
		CO_2 and H_2O (× 2)	39 ATP

(1) During anaerobic glycolysis through the EMP pathway (*e.g.*, yeast and homofermentative lactic acid bacteria) there is a net gain of only 2 molecules of ATP per molecule of glucose metabolised. (2) During glycolysis through the HMP pathway (*e.g.*, heterofermentative lactic acid bacteria) and the ED pathway (*e.g.*, *pseudomonas*) there is a net gain of only one molecule of ATP. (3) During alkaline fermentation in yeast no ATP is produced (Table 17.8).

When a molecule of glucose is completely metabolized to carbon dioxide and water under aerobic conditions, 38 molecules of ATP are produced : 8 during glycolysis, 6 during oxidation of pyruvate to acetyl CoA and 24 during the Krebs cycle.

In muscle glycolysis the initial substrate is not free glucose but *glycogen* (Fig. 17.35). Glycogen is phosphorylated by phosphoric acid and not by ATP. Thus instead of 2 ATP molecule only one is consumed. The net gain of ATP at the end of anaerobic glycolysis is therefore 4 – 1 = 3 ATP molecules. Under aerobic conditions 3 + 6 = 9 molecules of ATP are produced. The total ATP yield when glycogen is the initial source of glycolysis is therefore 39 molecules.

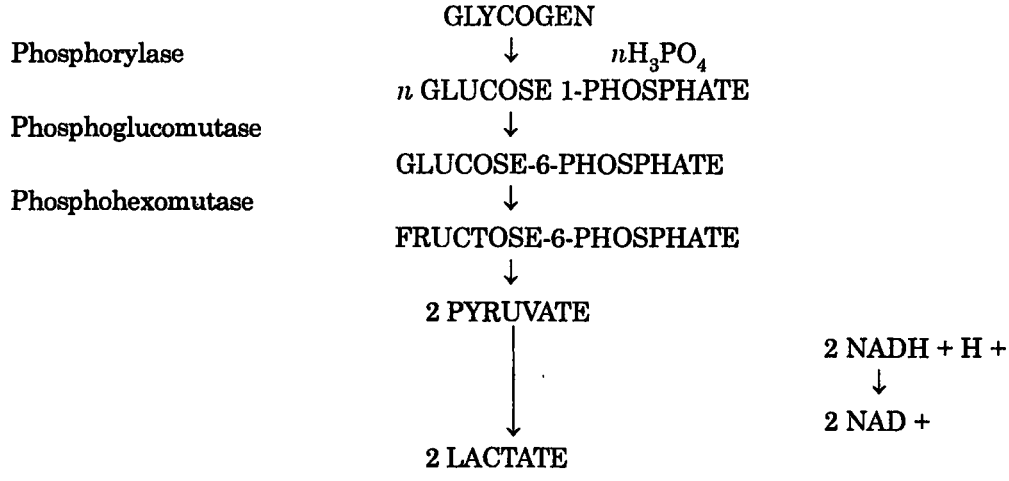


Fig. 17.35. Main steps in anaerobic muscle glycolysis.

In muscle glycolysis, lactic acid is produced under anaerobic conditions. Under aerobic conditons, glucose derived from glycogen is metabolized to carbon dioxide and water. However, recent work has shown that *even under fully aerobic conditions some lactic acid is produced.* Under anaerobic conditions lactic acid production is much larger, as much as 150 to 200 times more than the basal rate.

Under normal conditions the hydrogens released during respiration by muscle cells are accepted by oxygen, which is the final hydrogen acceptor. During violent physical exercise, as in fast running, the supply of oxygen is insufficient to accept all the released hydrogens. Pyruvate itself becomes the hydrogen acceptor and is transformed into lactate. In this case, as mentioned before, only 3 molecules of ATP are produced instead of the 39 under aerobic conditions. Accumulation of lactate results in muscle fatigue, and the runner may get cramps. When the runner stops running, he pants for some time, bringing oxygen to the muscles. In the presence of oxygen 1/5 of the lactate formed is metabolized to carbon dioxide and water, and energy is released as ATP. This energy is used in the resynthesis of the remaining 4/5 of lactate to glycogen.

Bacterial Electron Transport

In most bacteria, the respiratory chain is significantly different from the mitochondrial chain. The differences between electron transport systems in mitochondria and bacteria are as follows :

1. The electron transport chain of mitochondria is located in the *inner membrane*. In bacteria the chain is located in the *plasma membrane*. The similarity between the two membranes has led to the proposal of the *symbiotic theory* of the origin of mitochondria. According to this theory, primitive host cells which respired *anaerobically* by glycolysis were invaded by *aerobically* respiring bacteria like parasites. These invading organisms later came to live symbiotically in host cells and became the mitochondria. The inner-membrane of the mitochondrion is presumed to have evolved from the plasma membrane of the invading organism. A closer examination, however, shows that the similarity between the respiratory chains of bacteria and mitochondria is largely superficial. Most bacterial respiratory chains are significantly different from mitochondrial chains.

2. Bacterial respiratory systems2 have a great variety in their redox carrier patterns. One carrier may be replaced by another, and there may be addition or deletion of specific carriers.

3. Mitochondrial respiratory systems are two-dimensional, linear and almost unbranched. Bacterial chains are often three-dimensional and branched, with entry and exit of electrons at many points. Electrons may enter the chain through any of the several dehydrogenases, and leave by any of the several oxidases. Branched respiratory chains are of evolutionary importance. Because of the variable environment in which bacteria live, a mechanism for utilizing alternate electron acceptors is essential. Hence the branched chains. Such a device is unnecessary in mitochondria, which are situated in a controlled intracellular environment.

4. *Dehydrogenases :* Bacterial respiratory chains show extensive branching at the level of primary dehydrogenase. This enables them to directly oxidze a variety of substrates.

5. *Quinones :* Electron transfer from flavoproteins to cytochromes in mitochondria takes place through a mobile carrier which is *coenzyme* Q (CoQ, Q) or *ubiquinone* (UQ). Its reduced from is *hydroquinone* (CoH_2). CoQ has a side chain consisting of a number of *isoprenoid* units, the, number varying from 6-12 (CoQ_6—CoQ_{12}). In mammals the most common form is CoQ_{10}. In bacteria the side chain usually contains 7-10 isoprenoid units of 5 carbon atoms each. Thus there is a total of 35-50 carbons in the side chain.

In bacteria the quinone may be *coenzyme* Q or, *menaquinone* (MK) or *dimethyl menaquinone* (DMK).

Table 17.9
TYPES OF QUINONES IN BACTERIA
(Brackets indicate low concentration or activity)

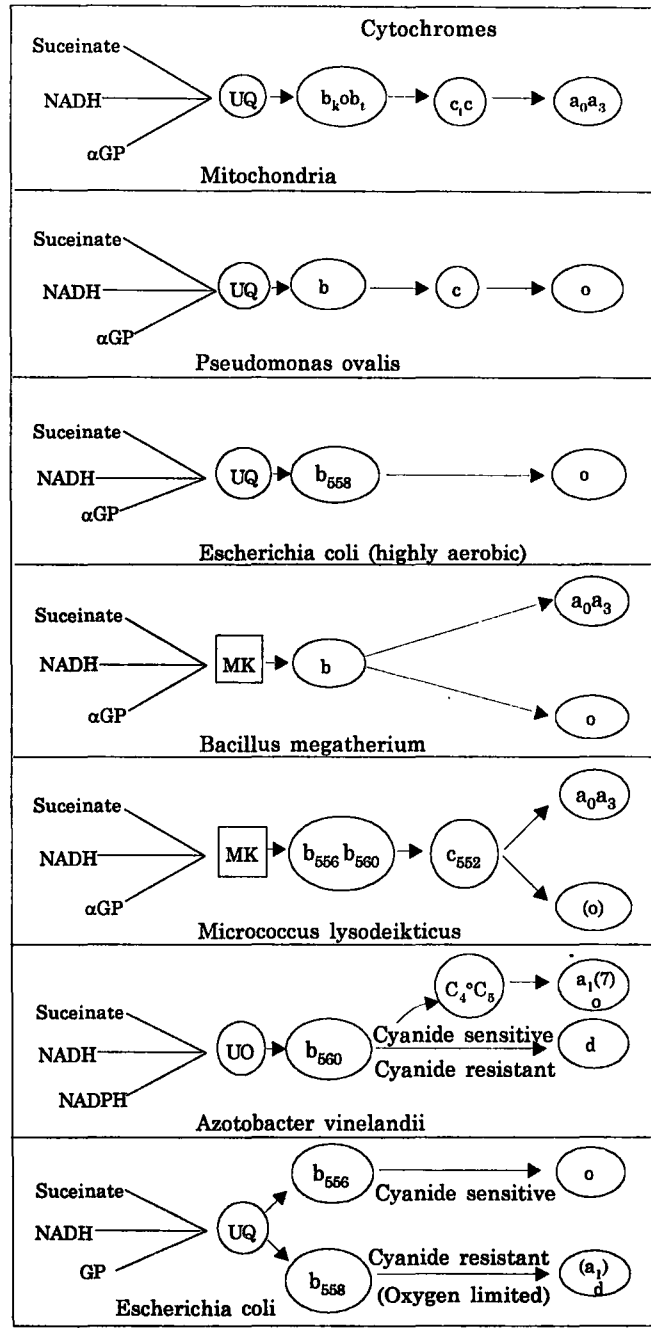
Bacterium		*Quinone*
Paracoccus denitrificans	CoQ	—
Micrococcus Lysodeikticus	—	MK
Pseudamonas ovalis	CoQ	
Haemophilus parainfluenzae	—	DMK
Azotobacter vinelandii	CoQ	—
Escherichia coli	CoQ	(MK)
Bacillus megatherium	—	MK

6. *Cytochromes :* Although basically similar to mammalian cytochromes, bacterial cytochromes possess properties not found in the former. Bacterial cytochromes show a greater variety of structure, function and location. Cytochromes *b*, *c*, *a*, *o* and *d* have been found in bacteria.

Cytochromes *b* in mammalian mitochondria are of two types, b_K (b_{560}) and b_T (b_{565}). Cytochromes b_{556}, b_{557} and b_{559} have been found in bacterial respiratory chains, and correspond to the b_K type. The b_T type cytochrome includes b_{560}, b_{561}, b_{562} and b_{563}.

Mitochondrial *cytochrome oxidase* ($a.a_3$) contains two haeme and two *copper* centres. *Haeme a* does not react with carbon monoxide, while *haeme* a_3 does. Because of this fact some workers have suggested that cytochrome oxidase consists of two different cytochromes, *a* and a_3. However since the two have never been separated they must be considered to be parts of the same complex. Cytochrome *a*. a_3 is present in some bacteria (*e.g., Bacillus megatherium* and *Micrococcus luteus*) and absent in others (*e.g., E. coli* and *Azotobacter vinelandi*). Other *a type* cytochromes present in bacteria (*e.g. Achromobacter* and *Azotobacter*) are a_1 and a_2 (?)

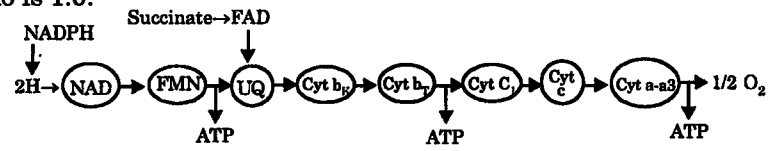
Cytochrome *o* has been reported in several bacteria. *Haemophilus parainfluenzae* and *Azotobacter vinel andii* have both cytochromes *o* and *d*. These are parts of terminal branches, of which one is more sensitive to cyanide than the other. In A, vinelandii the cyanide sensitive pathway consists of cytochromes *b*, c_4, c_5 and *o* (+ a_1 ?) and the *cyanide resistant* pathway $b \longrightarrow d$.



7. Oxidative phosphorylation : Synthesis of ATP in the presence of oxygen *is called oxidative phosphorylation. Inorganic phosphate* (P_i) takes in the energy released by oxidation of substrates and is converted into *energy-rich phosphate.* This phosphate is transferred to ADP and ATP is formed.

The number of moles of ATP formed relative to gram atoms of oxgyen consumed is called the P : O ratio. When $NADH_2$ is oxidized in mitochondria, 3 moles of ATP are formed per gram atom of oxygen consumed, *i.e.,* the P : O ratio is 3.0. As mentioned previously, one molecule of ATP is formed at each of three sites (I, II and III) when a pair of electrons passes down the

respiratory chain. When *succinate* is the acceptor, the first ATP-yielding site is bypassed, resulting in a P : O ratio of 2.0. When *lactate is the substrate,* ATP producing sites I and II are bypassed, and the P : O ratio is 1.0.



Heterotropic bacteria rarely show P : O ratios higher than 1.0 – 1.5 for oxidation of $NADH_2$. The more complex chemolithrotrophs sometimes show higher ratios. In many bacteria there are apparently only one or two coupling sites for oxidative phosphorylation. Most organisms utilize sites I and II during normal growth under aerobic conditions.

In vitro energy conservation at site *o* (*nicotinamide nucleotide transhydrogenase* TH) has been demonstrated in membrane vesicles of *Acinetobacter lwoffi* and *Pseudomonas ovalis.* ATP synthesis at this site is only possible in the presence of very high (NADPH) (NAD^+)/($NADP^+$) (NADH) ratio. There is no evidence that such a ratio is present under *in vivo* conditions. It is therefore unlikely that transhydrogenase plays a significant role in phosphorylation. In *Paracoccus denitrificans and Alcaligenes eutrophus* there appear to be four energy coupling sites. *Bacillus megatherium* and *Escherichia coli* contain only two or three sites. This is possibly because of their inability to synthesize transhydrogenase (Site *o*) and/or cytochrome *c* (Site III). In *Mycobacterium phlei,* three molecules of ATP are synthesized per molecule of oxidized substrate, through three separate respiratory chains.

8. Types of respiratory chains

(*i*) In some bacteria the respiratory chains have linear terminal pathways, as in mitochondrial respiratory chains. Unbranched terminal pathways are relatively rare and contain only one functional terminal oxidase (Fig. 17.36).

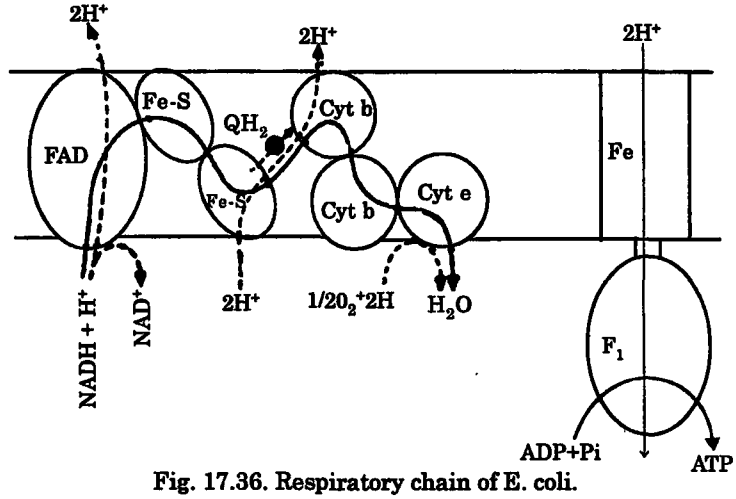


Fig. 17.36. Respiratory chain of E. coli.

(*ii*) Most bacterial respiratory chains show *terminal branching.* Such systems contain more than one functional cytochrome oxidase. Usually the branching occurs at the cytochrome *b* or cytochrome *c* level. There is simple electron transfer from cytochrome *b/c* molecular oxygen via oxidases.

(*iii*) In a few bacteria there are extensively branched terminal pathways involving cytochromes of the *b* and/or *c* type, as well as multiple cytochrome oxidases.

As mentioned previously, cytochrome oxidases *o* terminate *cyanide-sensitive* pathways, while cytochrome oxidase *b* terminate *cyanide-resistant* pathways. It will be seen that *Escherichia coli* has an *unbranched* terminal pathway under *highly aerobic* conditions, while during *oxygen-linited* growth the terminal pathway is branched.

III. Glyoxylate Cycle (Fig. 17.37)

The glyoxylate cycle is a modified version of Krebs cycle. It is an anabolic pathway which is present in certain microorganisms (*e.g.*, many bacteria, algae) and in certain higher plants at a particular stage of their life cycle. It is lacking in animals. The glyoxylate cycle is employed during growth on *acetate* and certain other substrates, *e.g., fatty acids.* Tissues of higher plants having the glyoxylate cycle have organelles called *glyoxysomes* which contain the five enzymes necessary for the cycle. Cotyledons of high-lipid seeds like castor and peanut contain glyoxysomes after germination, and are thus able to utilize lipid for carbohydrate synthesis.

Oxidation of acetate yields *acetyl CoA.* A molecule of *acetyl* CoA condenses with oxaloacetate to give *citrate.* Citrate is isomerized to *isocitrate* through *cis-aconitate,* as in the Krebs cycle. In the glyoxylate cycle the CO_2 producing steps of the Krebs cycle are bypassed. In this bypass two reactions convert *isocitrate* to *malate,* and one molecule of *acetyl* CoA is utilized. Isocitrate is cleaved by the enzyme isocitritase to produce succinate and *glyoxylate.* A molecule of acetyl CoA is utilized in the formation of glyoxylate. Glyoxylate, along with a molecule of water, yields *malate,* the reaction being catalysed by the enzyme *malate synthetase.* Malate is dehydrogenated by *malate dehydrogenase and oxaloacetate* is regenerated. The oxaloacetate is needed for the utilization of acetate.

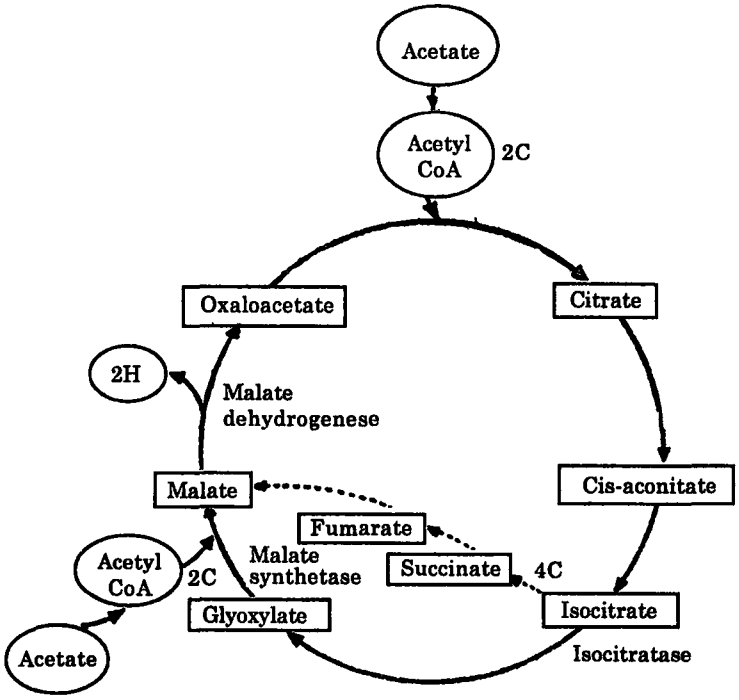


Fig. 17.37. The glyoxylate cycle.

In each turn of the cycle two molecules of *acetate* are utilized and one molecule of *succinate* is formed. The succinate can be utilized in several biosynthetic pathways, *e.g.*, (1) synthesis of

aspartic acid through oxaloacetate, (2) synthesis of *sugars,* and (3) synthesis of *porphyrins* through succinyl CoA.

Types of Respiration

Hydrogen atoms removed during glucose metabolism are accepted by NAD^+, which is reduced to $NAD + H^+$. NAD^+ must be regenerated from $NADH + H^+$ in order to pick up more hydrogen atoms. If this did not happen glycolysis would stop, resulting in the death of the organism. Living organisms have various mechanisms for disposing off hydrogen from $NADH + H^+$. The three main types of respiration are based on the manner in which organisms oxidize $NADH + H^+$ to NAD^+. In all these three types of respiration glycolysis (glucose to pyruvic acid) is the common feature. The fate of pyruvic acid and the method of hydrogen disposal determine the types of respiration, which are (1) fermentation, (2) anaerobic respiration by some bacteria, and (3) aerobic respiration.

(1) *Fermentation* takes place under anaerobic conditions, and is of two main types, *alcoholic fermentation* and *lactic acid fermentation, muscle glycolysis*) (Fig. 17.4). *Alcoholic fermentation* is carried on by yeasts, some bacteria, and even green plants under lack of oxygen. Pyruvic acid is first decarboxylated to *acetaldehyde,* which is then reduced to *ethyl alcohol* by NADH, and NAD^+ is regenerated.

$$\underset{C_6H_{12}O_6}{\text{Glucose}} \xrightarrow{\text{Glycolysis}} \underset{2C_3H_4O_3}{\text{Pyruvic acid}} \longrightarrow\longrightarrow \underset{2CH_3CHO}{\text{Acetaldehyde}} \longrightarrow \underset{2C_2H_5OH}{\text{Ethyl alcohol}}$$
$$\uparrow \quad \downarrow$$
$$NADH + H^+ \longrightarrow NAD^+$$

Lactic acid fermentation (*muscle glycolysis*) is characteristic of animals and some forms of bacteria (*e.g.,* lactic acid bacteria). Pyruvic acid formed as a result of glycolysis acts as a hydrogen acceptor and yields *lactic acid.* NAD^+ is regenerated. Both types of fermentation yield only two molecules of ATP.

$$\underset{C_6H_{12}O_6}{\text{Glucose}} \xrightarrow{\text{Glycolysis}} \underset{2C_3H_4O_3}{\text{Pyruvic acid}} \longrightarrow \underset{NADH + H^+ \longrightarrow NAD^+}{\text{Lactic acid}}$$

(2) *In anaerobic respiration* of some bacteria, hydrogen is passed on to oxygen which is not in the free form. It is found as a part of an inorganic compound such as sulphate (SO_4^{2-}) or nitrate (NO_3^-).

(3) *Aerobic respiration* takes place in the presence of free oxygen. As usual, pyruvic acid is first formed from glucose by glycolysis. Pyruvic acid is completely oxidized through the Krebs citric acid cycle and the hydrogen transport system to yield carbon dioxide and water. Oxygen serves as the final hydrogen acceptor. For each molecule of glucose completely oxidized to carbon dioxide and water, 38 molecules of ATP are generated.

$$\text{Glucose} \xrightarrow{\text{Glycolysis}} \text{Pyruvic acid} \xrightarrow[\text{Hydrogen transport system}]{\text{Krebs cycle and}} CO_2 + H_2O$$

Pathways in Intermediary Metabolism of Carbohydrate

The different pathways in the metabolism of carbohydrate are *glycogenolysis, glycolysis, Krebs citric acid cycle, gluconeogenesis* and the *pentose phosphate pathway* (Fig. 17.38)

(1) *Glycogenolysis :* The breakdown of glycogen is called *glycogenolysis.* In the liver the main end product of glycogen breakdown is *glucose.* In muscle the main products are *pyruvic acid* and *lactic acid.* Glycogen acts as a carbohydrates reserve. Whenever glucose is required, glycogen undergoes glycogenolysis, liberating glucose into the blood stream. The hormone *glucogon*

stimulates the conversion of glycogen to glucose in the liver, and thus causes a rise in glood sugar. Glucogen is secreted by the A-cells of the islets of Langerhans in the pancreas. *Epinephrine* (*adrenaline*) secreted by the medulla of the adrenal gland promotes conversion of glycogen to glucose in the liver, and also conversion of glycogen to lactic acid in muscle during anaerobic metabolism.

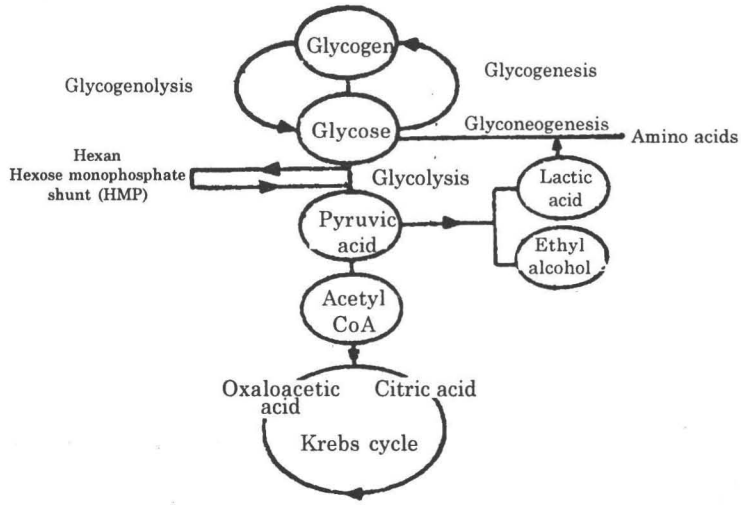


Fig. 17.38. Pathway in intermediary carbohydrate metabolism.

(2) *Glycogenesis* is the synthesis of *glycogen* (in animals) and *starch* (in plants) from glucose. When glucose is absorbed into the blood stream, some amount is immediately converted into glycogen in the liver. Some glucose passes from the liver into systemic circulation and is converted into glycogen in the muscles. An outline of glycogenesis is given below.

Glucose is converted into *glucose-6-phosphate,* which is, then converted into *glucose-I-phosphate.* Glucose-1-phosphate combines with *uridine triphosphate (UTP) to form uridine diphosphoglucose* (UDPG), with elimination of pyrophosphate (PPi).

Glucose-1-phosphate + UTP → UDP-glucose + pyrophosphate UDPG transfers the glucose molecule to the end of an existing glycogen chain. Repeated addition of one glucose unit at a time extends the glycogen chain.

UDP-glucose + Glycogen (n) → Glycogen (n + 1) + UDP. UDP can be reconverted to UTP transfer of phosphate from ATP.

$$UDP + ATP \rightleftharpoons UTP + ADP$$

(3) *Glycolysis :* The metabolic pathway starting with glucose and ending with pyruvic acid is called *glycolysis* (see Embden-Meyerhof-Parnas scheme).

(4) *Krebs citric acid cycle* is the final common pathway of oxidation of carbohydrates, proteins and fats through acetyl CoA which is completely oxidized to carbon dioxide and water.

(5) *Gluconeogenesis* is the formation of glucose or glycogen from non-carbohydrate precursors. The citric acid cycle and glycolysis are the main pathways of gluconeogenesis. The substrates are certain amino acids, lactic acid, glycerol and propionate. Gluconeogenesis from amino acids and lactic acid takes place mainly in the liver. Lactic acid formed in muscle is carried to the liver. Here it is converted into glucose by a reversal of the glycolysis mechanism, and stored as glycogen.

(6) *Pentose phosphate pathway* or *Hexose monophosphate (HMP) Shunt :* In the liver, adipose tissue and lactating mammary glands, there is an alternative pathway to the Embden-Meyerhof-Paranas scheme of glycolysis and the Krebs citric acid cycle for the oxidation of glucose to carbon dioxide and water. This pathway is called the *pentose phosphate pathway* or *hexose monophosphate shunt.* In this pathway for every six glucose molecules in the beginning, five hexoses are regenerated while one glucose molecule is oxidized.

18 Biosynthesis of Amino Acids

18.1 Introduction

Amino acids are of main importance in the metabolism of all organisms because they are the precursors of proteins. It is interesting to note that different organisms vary considerably in their ability to synthesise amino acids. For instance, man and the albino rat can synthesise 10 to the 20 essential amino acids required as building blocks of proteins. However, the remaining acids must be obtained from plants or bacteria. Higher animals can synthesise all the non-essential amino acids required as building blocks of proteins. However, the remaining acids must be obtained from plants or bacteria. Higher animals can synthesise all the non-essential amino acids only from ammonium ions but not from nitrite, nitrate or atmospheric nitrogen. On the other hand, higher plants can make all the amino acids required for protein synthesis from ammonia, nitrite or nitrate. Furthermore, the leguminous plants have symbiotic nitrogen-fixing bacteria in their root nodules and could use atmospheric nitrogen to synthesise amino acids.

Micro-organisms different in their capacity to synthesise amino acids. For example, *E. coli* could synthesise all their amino acids from ammonia while the bacterium *Leuconostoc mesenteroides cannot* grow unless it is supplied with a total of 16 different amino acids. Most micro-organisms need a reduced form of nitrogen (*e.g.*, ammonia) but there are numerous bacteria and fungi, as well as the higher plants which can utilise nitrite or nitrate.

The 20 different amino acids are synthesised by involving 20 different multienzyme sequences. The pathways of biosynthesis of amino acids are quite different from those employed in their degradation.

About 20 amino acids are commonly present in proteins. The synthesis of a number of these protein amino acids from the intermediates of glucose metabolism is indicated in Fig. 18.1 and Table 18.1.

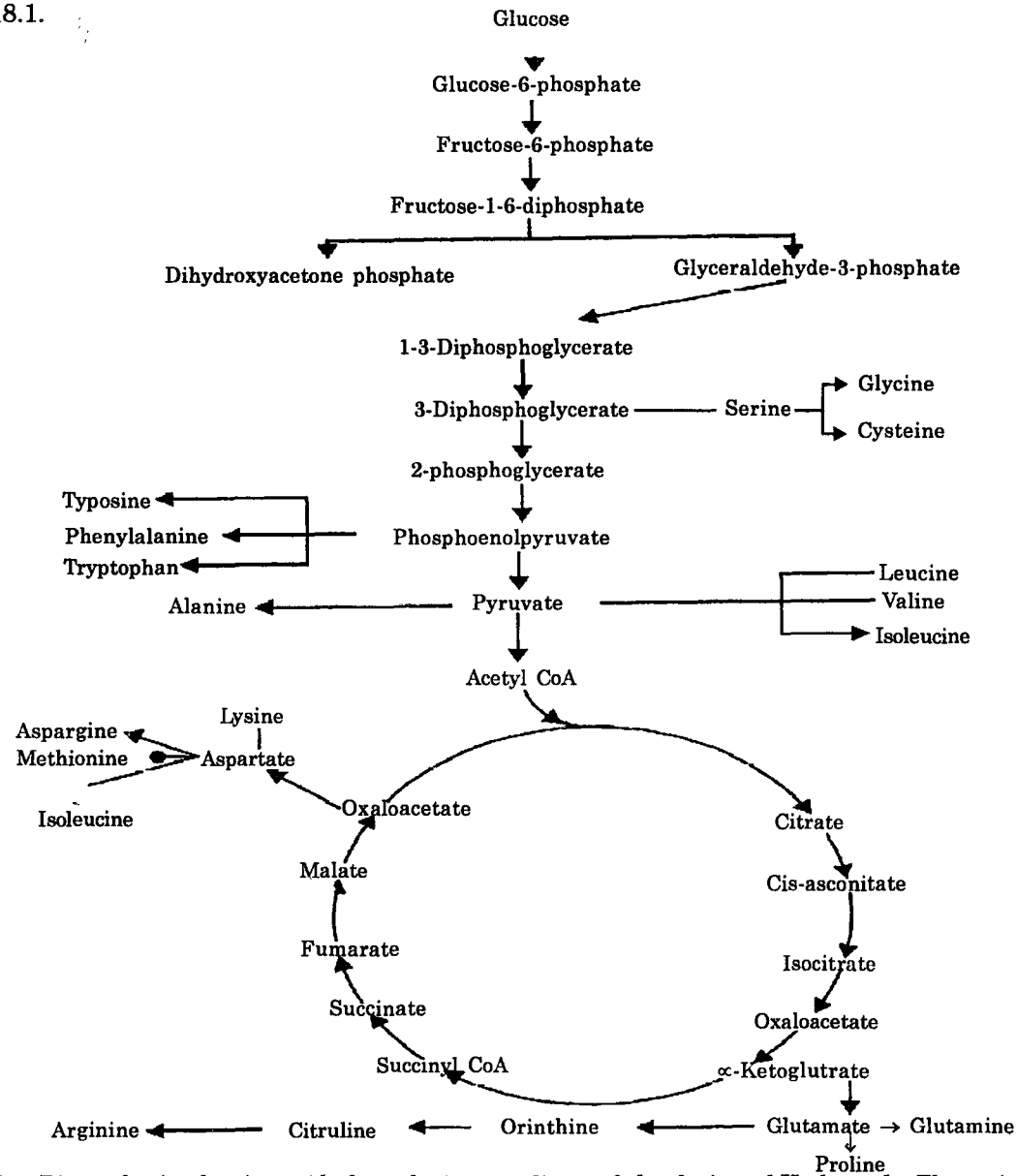


Fig. 18.1. Biosynthesis of amino acids from the intermediates of glycolysis and Krebs cycle. The amino acids citrulline and ornithine do not occur in proteins.

I. The Glutamate Family (Fig. 18.2)

The glutamate group of amino acid includes *glutamate, glutamine, proline, arginine* and *lysine* (in fungi). (Fig. 18.2).

1. Glutamate and glutamine : In most organisms, the main pathway for glutamate formation is from α-ketoglutarate. Glutamate is synthesised either by reductive amination with NH_4^+ or by transmination with aspartic or other amino donating groups.

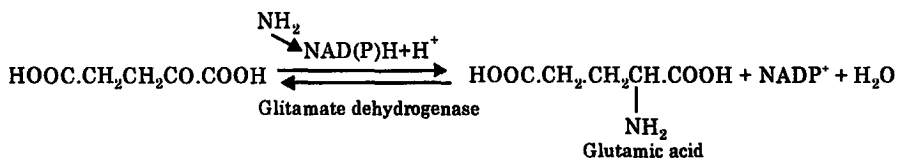
Table 18.1

BIOSYNTHESIS OF AMINO ACIDS

Family	*Precursors*	*Amino acids*
I. Glutamate family	α-Ketoglutarate—Glutamate	Glutamine Proline Arginine Lysine* (in fungi)
II. Serine family	3-Phosphoglycerate—Serine	Glycine Cysteine
III. Asparate family	Oxaloacetate—Aspartate	Asparagine Lysine* (bacteria, most alage, higher plants) Methionine Threonine Isoleucine**
IV. Pyruvate family	Pyruvate	Isoleucine** Valine Leucine Alanine
V. Aromatic amino acids	Phosphoenol pyruvate + Erythrose-4-phosphate	Tyrosine Phenylalanine Tryptophan
	Phosphoribosyl Pyrophosphate (PRPP) + ATP	Histidine

* The AAA pathway to lysine in fungi is being considered with the glutanmate family. The DAP pathway in bacteria, most algae and higher plants is being considered with the asparate family.

** Isoleucine is a member of the asparate family, but is being considered in the pyruvate family because four of its biosynthetic enzymes are also found in the valine pathway.



Or

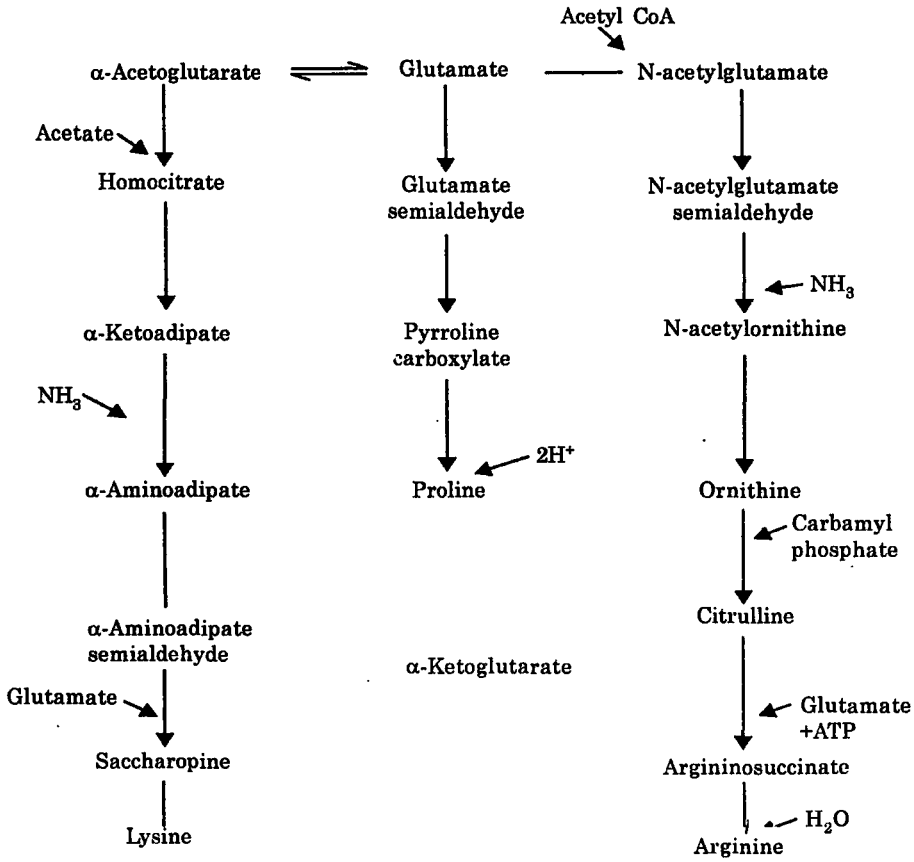


Fig. 18.2. Biosynthesis of glutamate family of amino acids.

Glutamate dehydrogense is a regulatory enzyme. In animal tissues it can utilise either NAD or NADP. In plants the enzyme is often specific for NADP.

Synthesis of glutamic acid by reductive amination has been found to be common in plants growing in soil. They get their inorganic nitrogen from the soil either in the form of NO_3^- or NH_4^+. Nitrate gets reduced to ammonium via nitrite and probably by hydroxylamine. The most important and probably the rate limiting enzyme in the pathway $NO_3 \longrightarrow NO_2 \longrightarrow NH_3 \longrightarrow$ amino acids is nitrate reductase whose activity has been regulated by various environmental and nutritional factors (Srivastva. H.S. 1980). Then ammonium enters the amino acid cycle through glutamic acid.

In plants, an alternative path for the incorporation of ammonium has been reported. The ammonium produced from the reduction of nitrate gets accepted by glutamic acid to form glutamine which, in turn, can bring about amination of α-ketoglutarate to two molecules of glutamate.

$$HOOC{-}CH_2{-}CH_2\underset{\displaystyle NH_2}{CH}{-}COOH + NH_3 \xrightarrow[\text{Glutamine synthetase}]{\text{ATP, } Mg^{2+}} H_2NOC\,CH_2CH_2\underset{\displaystyle NH_2}{CH}\,COOH$$

Glutamate — Glutamine

$$H_2NOC{-}CH_2{-}CH_2{-}\underset{\displaystyle NH_2}{CHCOOH} + HOOC.CH_2CH_2CO.COOH$$

Glutamate — α-Ketoglutarate

$$\xrightarrow[\text{Glutamate synthase}]{2H} 2\,HOOCCH_2CH_2CH_2CHNH_2.COOH$$

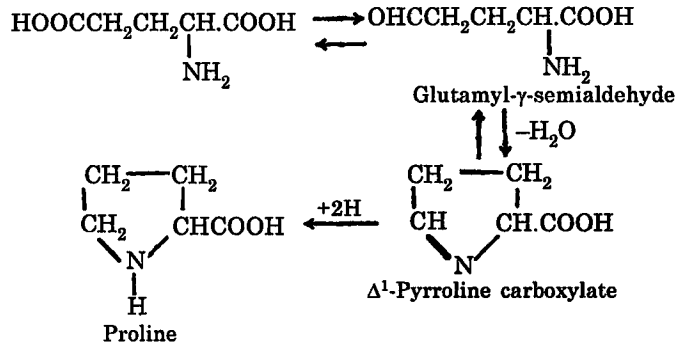
Glutamate

The enzymes glutamine synthetase and glutamate synthase have been isolated and characterised in various enzymes. Glutamine synthetase is a structurally complex enzyme of molecular weight 350,000 to 600,000 depending on the species. It is postulated that assimilation of ammonia in the symbiotic nitrogen fixing systems involves glutamate synthase pathway.

Srivastva, H.S. 1980, Regulation of nitrate reductase in higher plants. Phytochem. 19 : 725.

Glutamic acids synthesised may enter into proteins. This acid also forms the source of carbon atoms of proline and arginine.

2. Proline : Proline is synthesised from glutamic acid. In this synthesis, glutamic acid is first reduced to an aldehyde called glutamyl-α-semialdehyde. This compound in aqueous solution remains in equilibrium with acyclic compound Δ-pyrroline carboxylate which is finally reduced to proline.



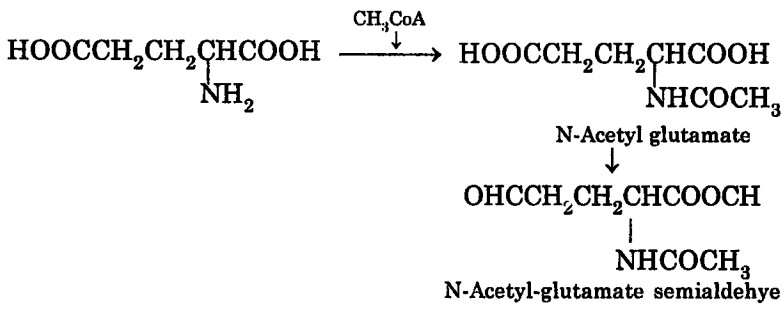
Those reactions have been reported in many organisms and also in pea plants. Those have been summarised as below :

$$\text{Glutamic acid} + \text{ATP} + \text{NADH} \xrightarrow[\text{Glutamyl-}\gamma\text{-semialdehyde} + \text{ADP} + \text{NAD}^+ + \text{Pi}]{\text{Glutamate kinase and dehydrogenase}}$$

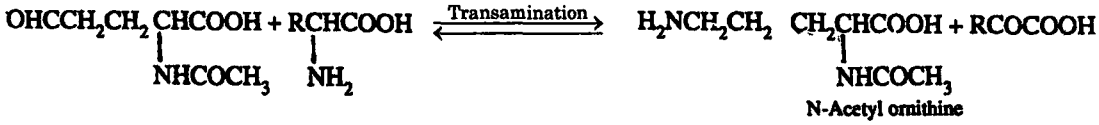
$$\text{Glutamyl-}\gamma\text{-semialdehyde} \xrightarrow{-H_2O} \Delta^{1}\text{- Pyrroline carboxylate}$$

$$\Delta^{1}\text{-Pyrroline carboxylate} + \text{NADPH} \xrightarrow[\text{Proline} + \text{NADP}^+]{\text{Pyrroline-5-carboxylate reductase}}$$

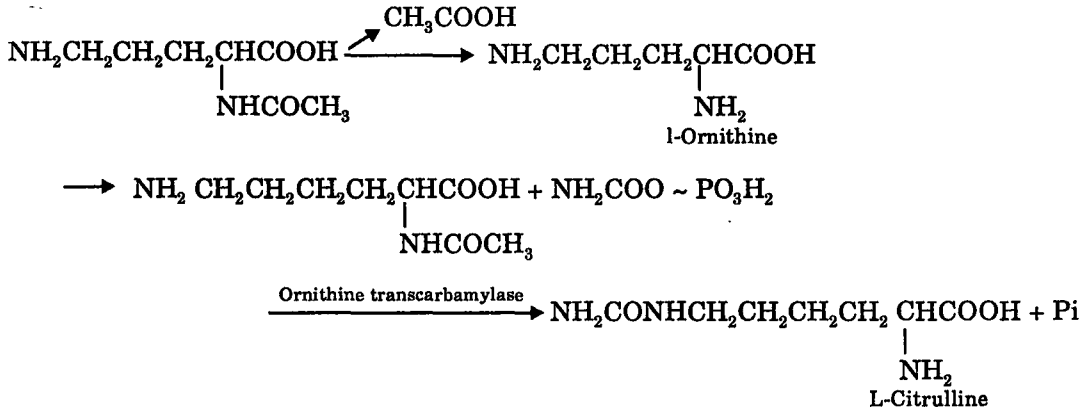
3. Arginine and ornithine : Arginine gets synthesized from glutamic acid by involving acylation and transamination processes. In the first step, a specific acetylase has been found to catalyse the conversion of glutamic acid to N-acetyl-glutamic acid which then undergoes reduction to form N-acetyl-glutamate semialdehyde.



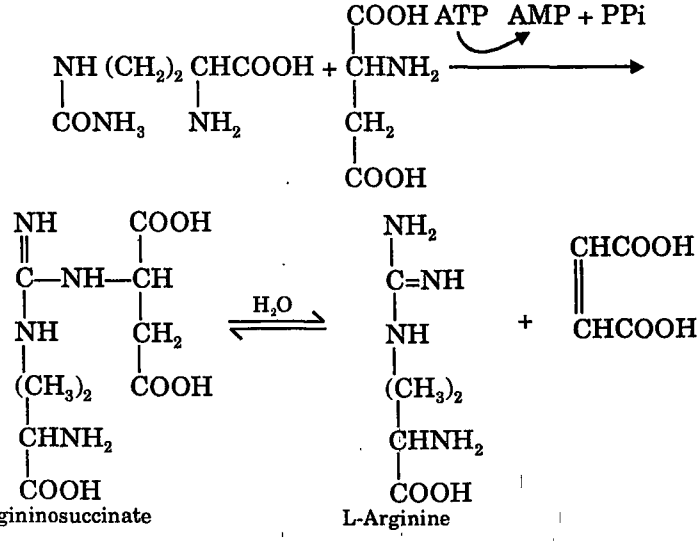
Acetylation of this aldehyde at—NH_2 group does not allow cyclization (as it is seen in the synthesis of proline). The N-acetyl-glutamate semialehyde gets transaminated to form N-acetyl-ornithine.



The amino acid acetyl-ornithine is widely found in plants. In some organisms, the removal of N-acetyl group is done by a specific deacylase, but in others it gets transferred again to the amino group of glutamate. Deacylation yields L-ornithine which then gets converted to L-citrulline, by employing one molecule of carbamyl phosphate.

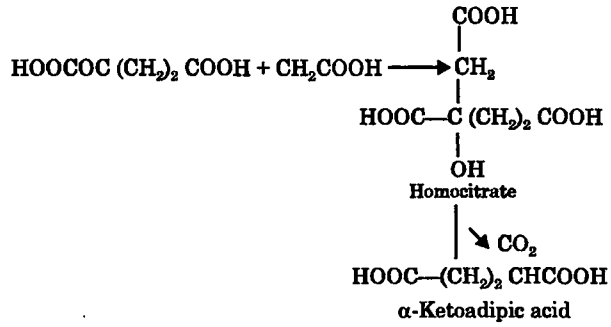


Arginine is synthesised from citrulline by involving aspartate as amino donor and argininosuccinate as an intermediate product. Energy derived from ATP is used in the synthesis of argininosuccinate. Argininosuccinate then gets hydrolysed to form arginine and fumaric acid by the enzyme called argininosuccinase. The presence of this enzyme, and the one which is synthesising argininousuccinate has been reported in micro-organisms and plants.

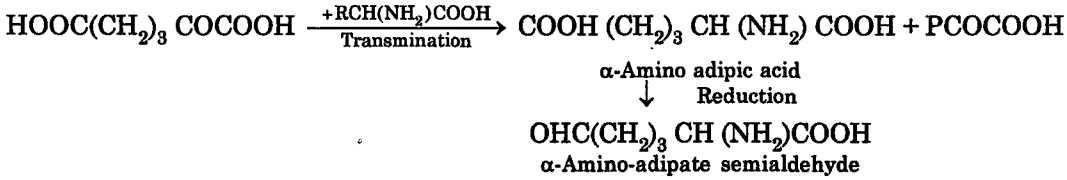


4. Lysine : Synthesis of lysine in some fungi takes place by involving a-ketoglutaric acid. This acid undergoes condensation with acetate to form homocitrate, which then gets isomerized

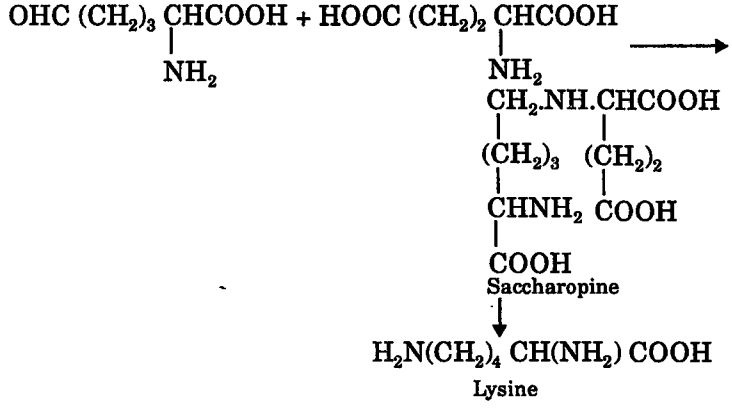
to form homoisocitrate. Homo-isocitrate then gets oxidized and decarboxylated to yield α-ketoadipic acid.



It is to be remembered that α-ketoadipic acid is the homologue of α-ketoglutaric acid. It gets transaminated to form α-amino-adipic acid, which then gets reduced to form α-amino adipate semialdehyde. This reduction is somewhat similar to the reduction of glutamic acid.



This semialdehyde undergoes condensation with a new molecule of glutamate to form saccharopine which, then gets converted to lysine.



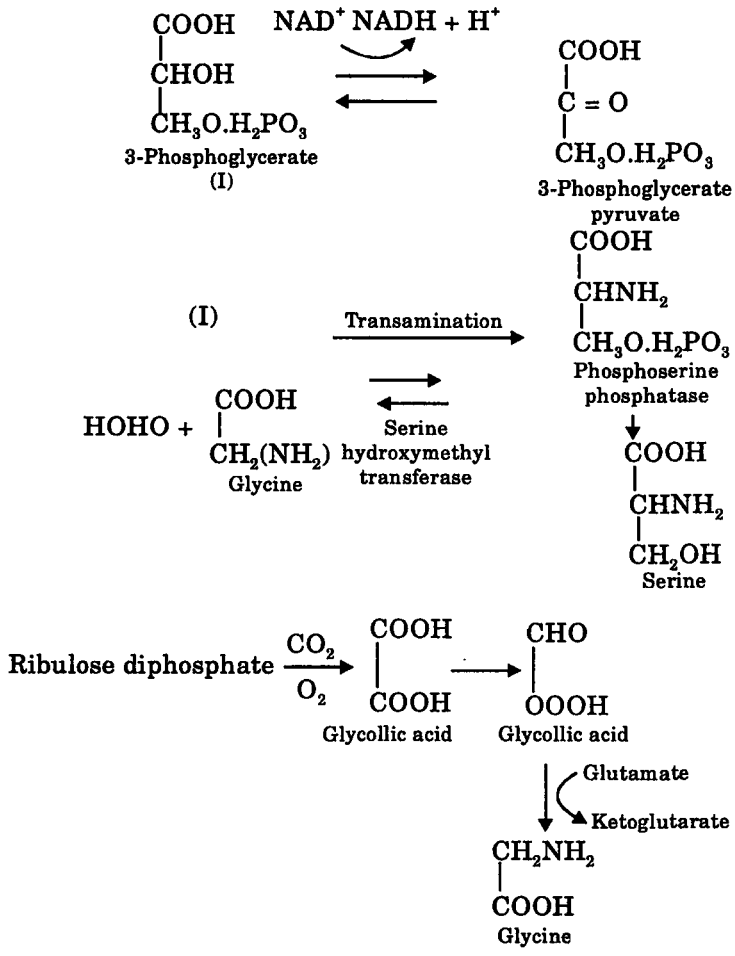
II. The Serine Family

The serine family of amino acids includes *serine, cysteine* and glycine. Cysteine and glycine are derived from serine.

1. Serine and glycine : In glycolysis, the intermediate is phosphoglycerate which is the parent compound for the synthesis of both amino acids, serine and glycine.

The conversion of serine to glycine is reversible and in photosynthetic tissues, glycine may be the source of serine biosynthesis.

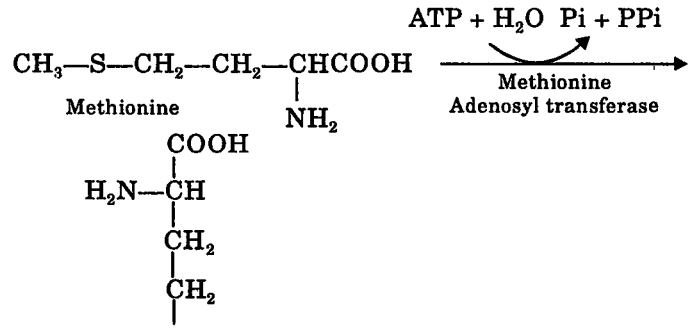
The plants having C_3 pathway (Calvin cycle) of photosynthesis form large amount of glycollic acid in high light intensities, low CO_2 and high O_2 concentrations. A part of glycollic acid gets converted to glycine with glutamate.

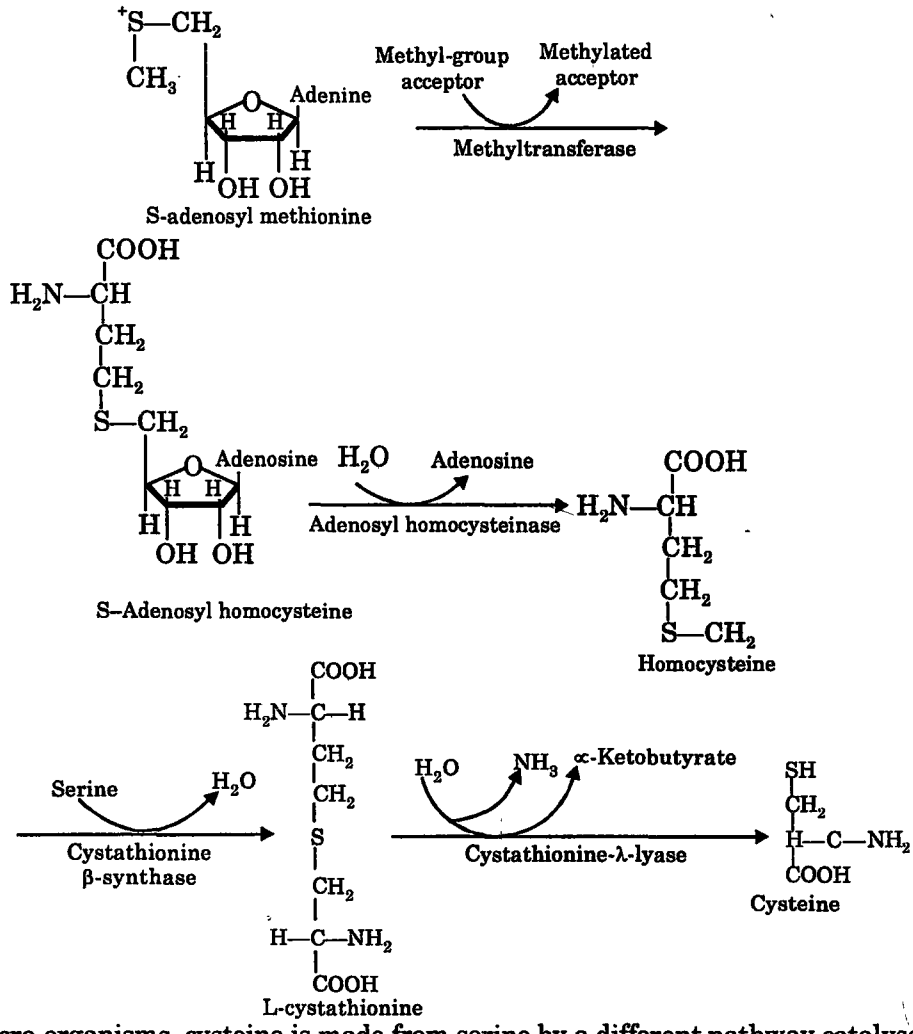


Glycine may also be synthesised from L-serine by the action of serine hydroxymethyl-transferase which catalyses transfer of the β-carbon atom of serine to tetrahydrofolate to yield N^5, N^{10}-methylene-tetrahydrofolate.

L-serine + Tetrahydrofolate $\rightleftharpoons$ Glycine + N^5, N^{10}-Methylene-tetrahydrofolate

2. Cysteine : Although cysteine is not an amino acid, it arises in mammals from methionine (which is essential) and serine (which is not-essential). In this pathway, the sulphur atom of methionine is transferred to replace the hydroxyl oxygen atom of serine, thereby converting serine into cysteine. This process is often termed as trans-suphuration.





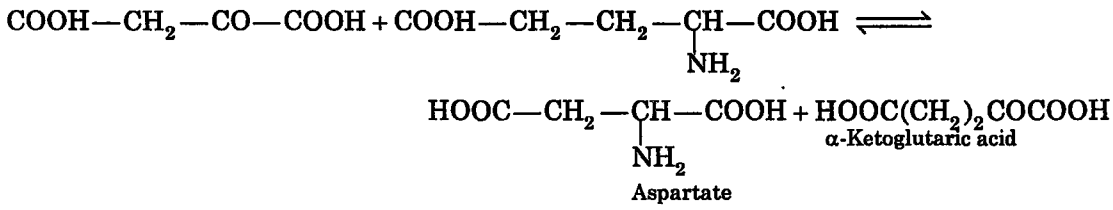
In some micro-organisms, cysteine is made from serine by a different pathway catalysed by cysteine synthase, a pyridoxal phosphate enzyme.

$$\text{L-serine} + H_2S \rightleftharpoons \text{L-cysteine} + H_2O$$

III. The Aspartate Family (Fig. 18.3)

The asparate family includes *aspartate, asparagine, methionine, threonine, lysine* (except in fungi) and *isoleucine.* Although lysine and isoleucine are included in the aspartate family, a part of their carbon skeletons is derived from pyruvate. The biosynthesis of isolecine has been described along with that of the pyruvate family, because its enzymes are also required in valine biosynthesis.

1. Aspartate : In most organisms, aspartate arises from oxaloacetate by transamination from L-glutamate.



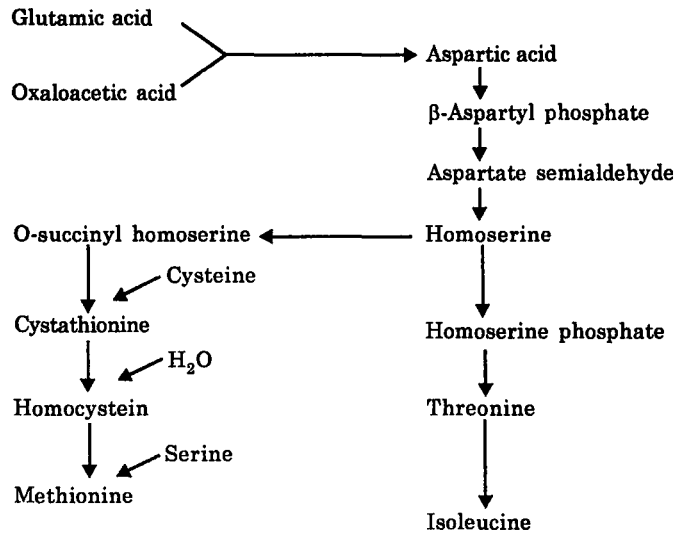


Fig. 18.3. Summary of biocynthesis of amino acids of the aspartate family.

The fumarate, which is another intermediate of Kreb's cycle, may also produce asparate by direct amination.

$$COOH—CH=CH—COOH + NH_3 \rightleftharpoons COOH—CH_2—CH(NH_2—COOH)$$

The enzyme *aspartase,* which catalyses this reaction, has been found to occur in E. coli and other micro-organisms. This reaction is readily reversible and deamination of aspartate to fumarate takes place readily.

2. *Asparagine :* Aspartic acid is the direct precursor of asparagine in a reaction catalysed by asparagine synthetase, which is analogous in its mechanism to glutamate synthetase.

$$HOOC—\underset{\displaystyle NH_2}{\underset{|}{CH_2}}—CH—COOH + NH_3 + ATP \longrightarrow H_2N—\overset{\displaystyle O}{\overset{\|}{C}}—CH_2—\underset{\displaystyle NH_2}{\underset{|}{CH}}—COOH + ADP + Pi$$

Aspartate — Asparagine

In some organisms an alternative pathway may occur in which the amide amino group of glutamine is transferred to the β-carboxyl group by the action of asparagine synthetase (glutamine hydrolysing).

$$H_2NOC—CH_2—CH_2—\underset{\displaystyle NH_2}{\underset{|}{CH}}COOH + HOOC—CH_2—\underset{\displaystyle NH_2}{\underset{|}{CH}}—COOH \longrightarrow \text{(+ATP)}$$

Glutamine — Aspartate

$$HOOC—CH_2—CH_2—\underset{\displaystyle NH_2}{\underset{|}{CH}}—COOH + H_2N—\overset{\displaystyle O}{\overset{\|}{C}}—CH_2—\underset{\displaystyle NH_2}{\underset{|}{CH}}—COOH$$

Glutamate — Asparagine — +AMP+PPi

3. Lysine : β-aspartic semialdehyde condenses with pyruvate to yield, 2, 3-*dihydrodipicolinate,* which cyclizes spontaneously. 2, 3-*Dihydrodipocolinate* is then reduced in an NADPH-linked reaction to yield Δ' *piperideine-2-dicarboxylate.* Succinylation (in *E. coli*) or acetylation (in bacilli) are involved in ring opening. As transamination reaction involving glutamate results in the formation of a *succinyl or acetyl diaminopimelate.* Removal of the acyl group results in the formation of *L, L-diaminopimelate.* By the action of a specific racemase, the *meso* form

diaminopimelate is obtained. Either L, L-diaminopimelate or the *meso* form, or both, depending upon the organisms, are used in the synthesis of the bacterial cell wall. A decarboxylase catalyses the *meso* form to yield *lysine.*

4. Methionine : Aspartic acid gets converted to β-aspartyl semi-aldehyde by involving its phosphate.

$$HOOCCH_2CH(NH_2)COOH + ATP \xrightleftharpoons{\beta\text{-asparto kinase}} \underset{\beta\text{-Aspartyl phosphate}}{H_2O_3POOCCH_2CH(NH_2)COOH} + ADP$$

$$H_2O_3POOCCH_2CH(NH_2)COOH \xrightleftharpoons{NADPH\ NADP^+} \underset{\beta\text{-Aspartyl semialdehyde}}{OHCCH_2CH(NH_2)COOH} + Pi$$

It has been reported that the common intermediate is β-aspartyl semialdehyde in the synthesis of methionine, threonine and isoleucine. Homoserine is the branching point for carrying out the synthesis of these amino acids. Homoserine gets synthesized by the reduction of aspartyl phosphate in the presence of the enzyme called homoserine dehydrogenase. The dehydrogenase may use either NADH or NADPH as coenzyme.

$$OHCCH_2CH(NH_2)COOH + NADPH + H^+ \rightleftharpoons \underset{\text{Homoserine}}{HOH_2CCH_2CH(NH_2)COOH} + NADP^+$$

In higher plants it has been reported that homoserine undergoes condensation with a cysteine molecule to form homocysteine, which yields methionine after methylation. However *E. coli* and *Neurospora crassa,* involve the complex synthesis of homocysteine. Alcoholic group of homoserine undergoes acylation first either with succinyl CoA or acetyl CoA to form either O-succinyl or O-accetyl-homoserine.

$$HOH_2CCH_2CH(NH_2)COOH + \begin{matrix}CH_2COOH\\ |\\ CH_2COOH\end{matrix} \longrightarrow \underset{\text{O-Succinyl-homoserine}}{HOOC(CH_2)_2COO(CH_2)_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCOOH}$$

These products on reacting with cysteine form cystathionine, which then gets hydrolyzed to form homocysteine, pyruvate and ammonia.

$$HOOCCH(NH_2)CH_2.SH \rightarrow HOOC(CH_2)_2COO(CH_2)_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCOOH \longrightarrow HOOCCH\underset{\displaystyle NH_2}{\underset{|}{C}}H_2S(CH_2)_2CH(NH_2)COOH \xrightarrow{H_2O} HS.CH_2CH_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCOOH + CH_2CO.COOH + NH_3$$

Homocysteine

As in higher plants, homocysteine gets methylated at—SH end and the product of the reaction has been found to be methionine. The methyl donor has been found to be another amino acid, serine.

$$HSCH_2CH_2CH(NH_2)COOH \xrightarrow{Serine} \underset{\text{Methionine}}{CH_3SCH_2CH_2CH(NH_2)COOH}$$

5. Threonine : Homoserine obtained from aspartate through aspartylsemialdehyde has been reported to be the precursor for biosynthesis of threonine also. Actually threonine is an isomer of homoserine in which alcohol group is occupying secondary position.

$$\underset{\text{Homoserine}}{HOOC.CH(NH_2)CH_2CH_2CH_2OH} \longrightarrow \underset{\text{Threonine}}{HOOC.CH(NH_2)CHOHCH_3}$$

The homoserine is converted into threonine by involving two steps. In the first step, the —OH group of homoserine gets phosphorylated in the presence of enzyme called homoserine kinase. Then this product is attacked by the enzyme homoserine phosphate metaphosphatase or O-Phosphohomoserine lyase, for transferring alcohol group to the secondary position. The enzyme is also named as threonine synthase, because the product is L-threonine.

$$HOH_2C.CH_2CH(NH_2)COOH + ATP \xrightarrow{\text{Homoserine kinase}} \underset{\text{Homoserine phosphate}}{H_2O_3P.O.CH_2CH_2CH(NH_2)COOH} + ADP$$

Homoserine phosphate metaphosphatase

$$CH_3CHOHCH(NH_2)COOH + Pi$$

Threonine

In micro-organisms, isoleucine gets synthesized from threonine which is first of all deaminated by biosynthetic threonine deaminase to form α-ketobutyric acid then α-ketobutyric acid undergoes condensation with acetaldehyde derived from pyruvate to form isoleucine finally after undergoing a series of reaction. Details of this pathway have been given later.

IV. The Pyruvate Family (Fig. 18.4)

Alanine, valine and *leucine* derive the major part of their carbon skeleton from pyruvate. *Isoleucine* is a member of the aspartate family which obtains two of its six carbons from pyruvate. It will be considered along with *valine,* since biosynthesis of the two amino acids requires basically a common set of enzymes. *Lysine,* another member of the aspartate family, can also derive half its carbon from pyruvate.

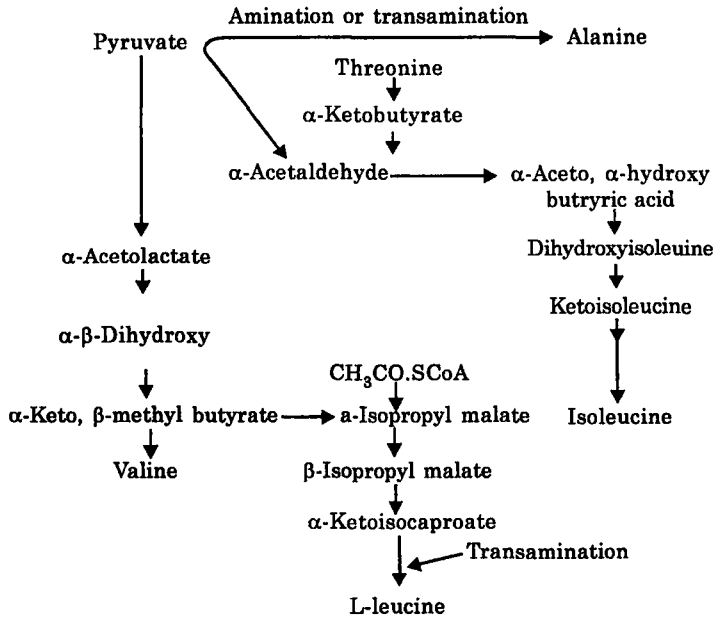
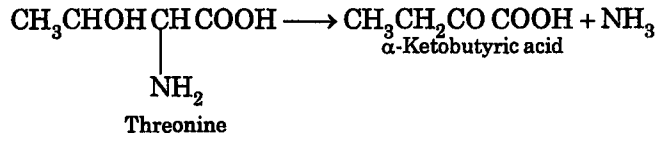


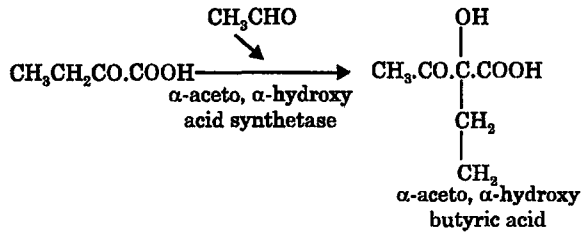
Fig. 18.4. Summary of pyruvate family amino acid synthesis.

Reactions involving the synthesis of amino acids derived from pyruvate are depicted in Fig. 18.4.

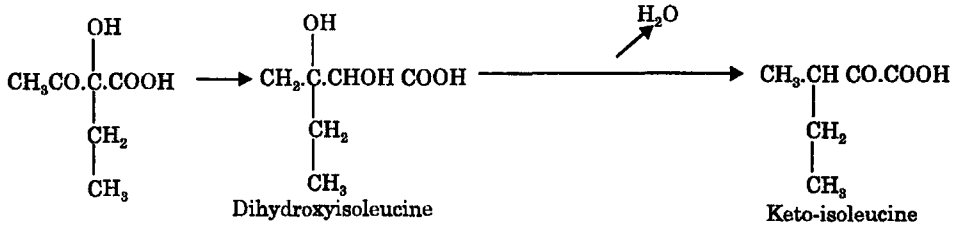
1. Isoleucine : Synthesis of isoleucine from pyruvate needs another amino acid called threonine. Threonine is first of all deaminated by threonine deaminase to form α-ketobutyric acid.



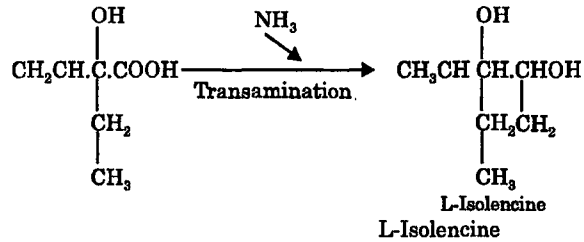
α-Ketobutyric acid then undergoes condensation with a molecule of acetaldehyde derived from pyruvate to form α-aceto, α-hydroxybutyric acid. The enzyme called α-aceto, α-hydroxy acid synthetase has been found to be the same which is catalysing the synthesis of α-acetolactate from pyruvate in valine biosynthetic pathway.



The α-aceto, α-hydroxy butyric acid gets rearranged to form dihydroxy isoleucine which then gets degraded to yield ketoisoleucine.

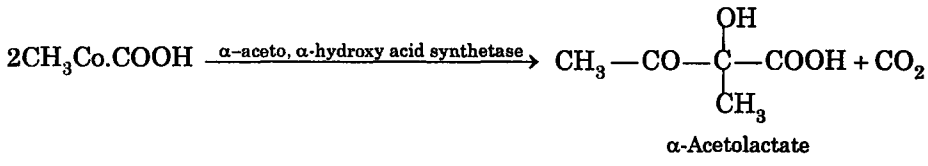


Ketoisoleucine then undergoes transamination to form isoleucine.

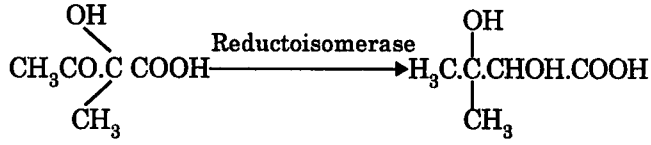


L-Isolencine

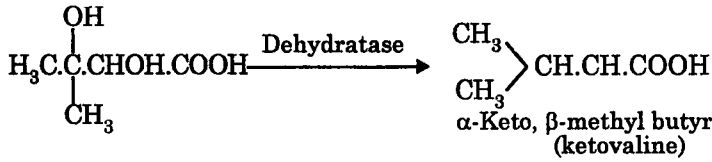
2. Valine and leucine : Knowledge about the synthesis of valine and leucine from pyruvate is mainly derived from investigations done on *E. coli* and other micro-organisms. An acetaldehyde molecule obtained from pyruvate undergoes condensation with another molecule of pyruvate to form α-acetolactate in the first step.



The product gets rearranged by the enzyme reductoisomerase involving the reduction of carboxyl group.

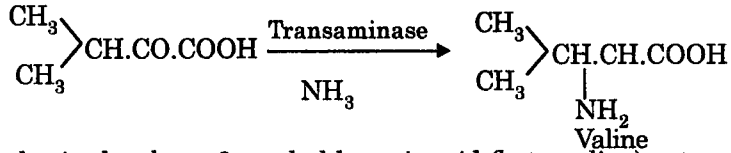


Then, α-β-dihydroxy β-methyl-butyrate gets dehydrated subsequently to form α-keto, β-methyl-butyric acid.



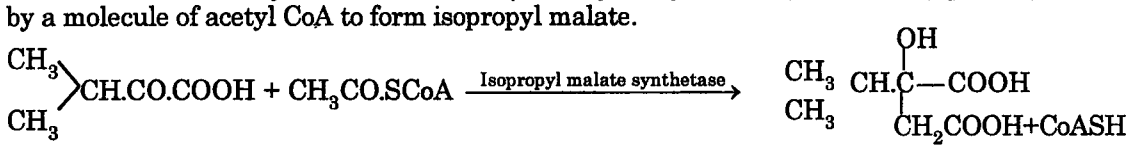
α-Keto, β-methyl butyric acid (ketovaline)

Ketovaline has been reported to be a precusor for both valine and leucine. When it gets transaminated it forms valine.



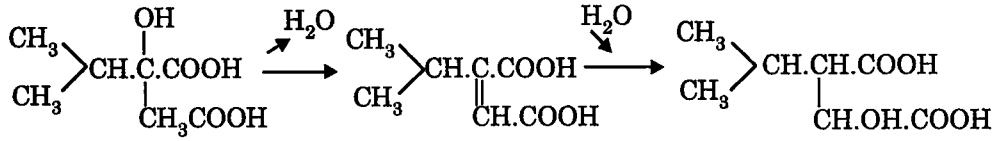
Valine

When leucine is synthesised, α-keto, β-methyl-butyric acid (keto-valine) gets acylated first by a molecule of acetyl CoA to form isopropyl malate.



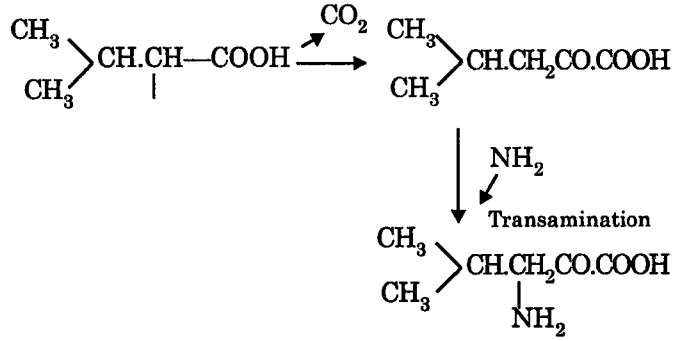
α-Isopropyl-malate

Then, α-isopropyl-malate undergoses rearrangement to form β-isopropyl-malate. Actually, this rearrangement takes place into two steps. In first step, the molecule gets dehydrated and in the next, it gets hydrated again.



β-Isopropyl-malate

β-Isopropyl-malate on oxidative decarboxylation forms α-keto-isocarporate (ketoleucine) which then gets transaminated to form leucine.

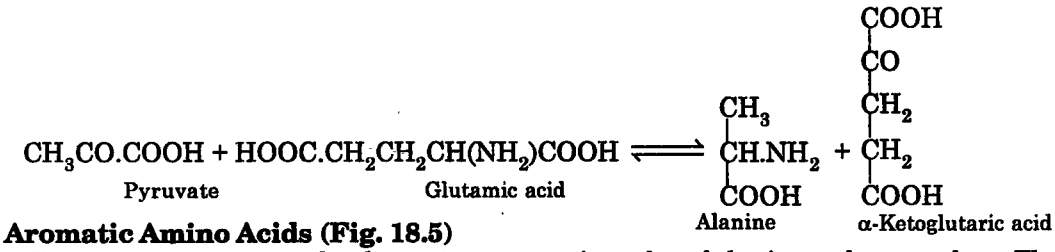


3. Alanine : In several micro-organisms, reductive amination of pyruvate yields alanine.

$$CH_3CO.COOH + NH_3 \xrightleftharpoons{NADH + H^+ \; NAD^+} CH_3CH(NH_2)COOH + H_2O$$

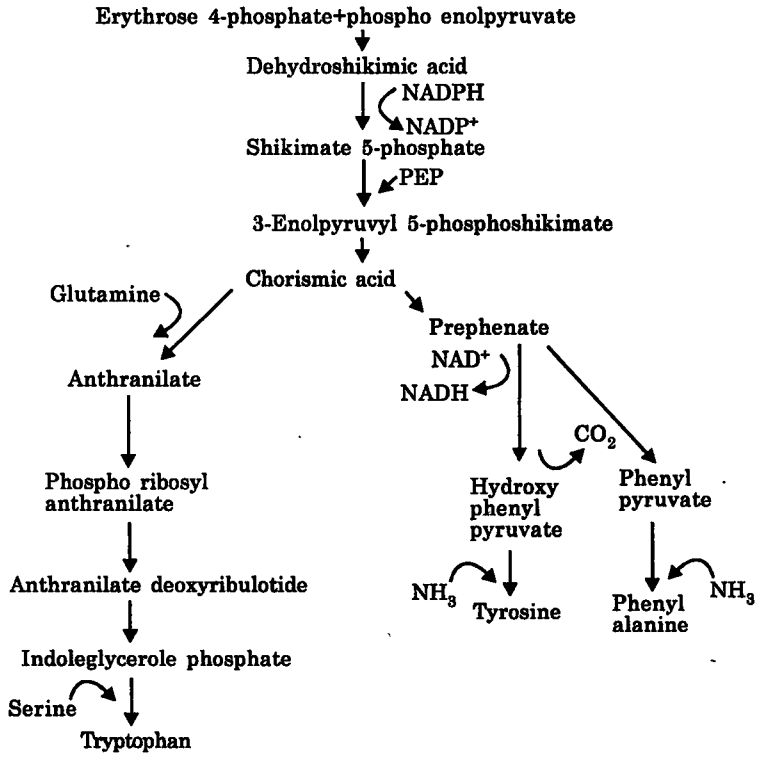
Pyruvate — Alanine

In other living organisms, it is evident that alanine gets synthesised by a transamination reaction.



V. Aromatic Amino Acids (Fig. 18.5)

The aromatic amino acids of protein are *tyrosine, phenylalanine* and tryptophan. There is a common pathway leading to the synthesis of chorismate, from which pathways branch to the three amino-acids. The pathways to phenylalanine and tyrosine are completely separated in some microorganisms, while in others they share an additional enzyme.

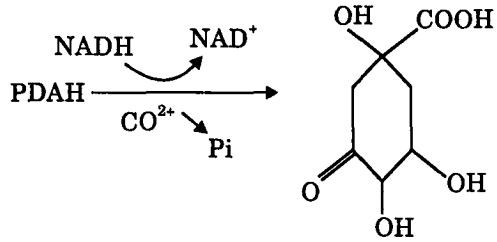


1. Chorismic acid : The knowledge about the biosynthesis of aromatic amino acids is mainly derived from investigations on micro-organisms. Shikimic acid, which occurs in plants as a rare chemical, has been found to be precursor for tyrosine, phenylalanine and tryptophan and many other aromatic compounds. It is possible to synthesise shikimic acid from two common compounds

of glucose degradation, erythrose 4-phosphate and phosphoenol-pyruvate. A specific aldolase brings about their condensation to yield 7-phospho, 3-deoxy, D-arabino heptulosonic acid (PDAH).

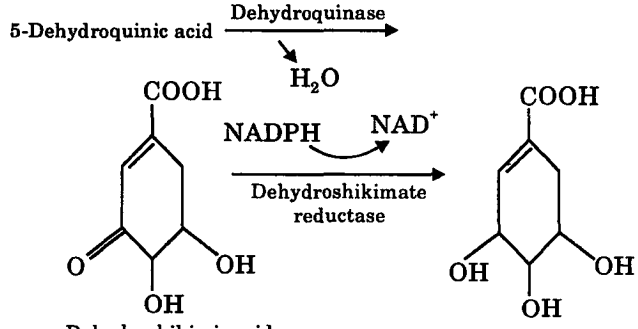
$$HOOCCO{-}P = CH_2 + OHC.CHOH.CHOH.\ CH_2O{-}P \xrightarrow{Pi} \underset{PDAH}{HOOCCO.CH_2(CHOH)_3CH_2O{-}P}$$

In the presence of NADH and CO^{2+}, PDAH gets reduced to 5-dehydroquinic acid with one or more enzymes. Dehydroquinic acid is a cyclic compound which does not have double bonds and therefore has no aromatic properties.



5-Dehydroquinic acid

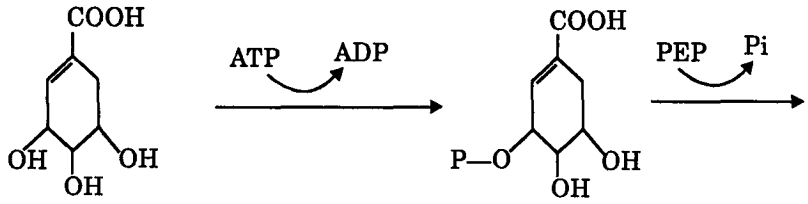
The dehydroquinic acid loses a water molecule to form dehydroshikimic acid which gets reduced with NADPH to form finally shikimic acid.



Dehydroshikimic acid

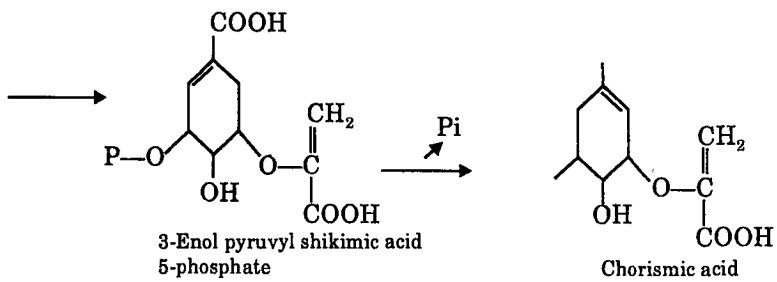
Shikimic acid

When chorismic acid is synthesised from shikimic acid, the later gets phosphorylated first to form phosphoshikimic acid which then reacts with one molecule of phosphoenolpyruvate to form 3-enol pyruvylshikimic acid 5-phosphate. This is then dephosphorylated to form chorismic acid.



Shikimic acid

5-Phosphoshikimic acid

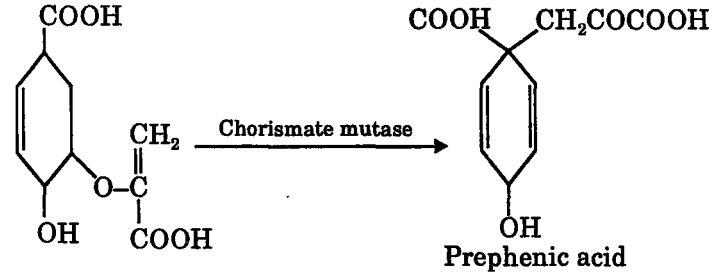


3-Enol pyruvyl shikimic acid 5-phosphate

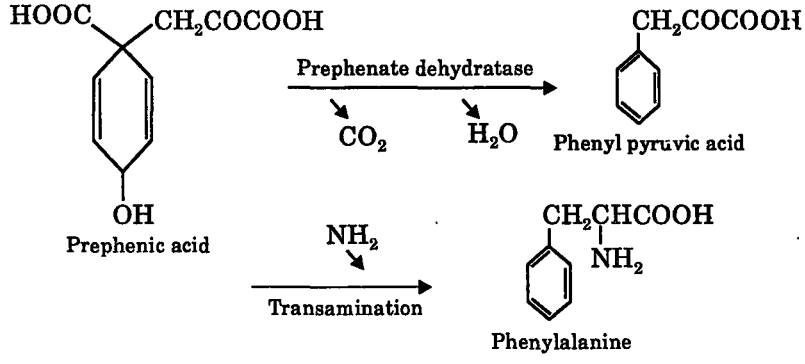
Chorismic acid

The name chorismic acid is derived from a Greek word which means separation. It is the product where biosynthetic pathways of amino acids get diverged.

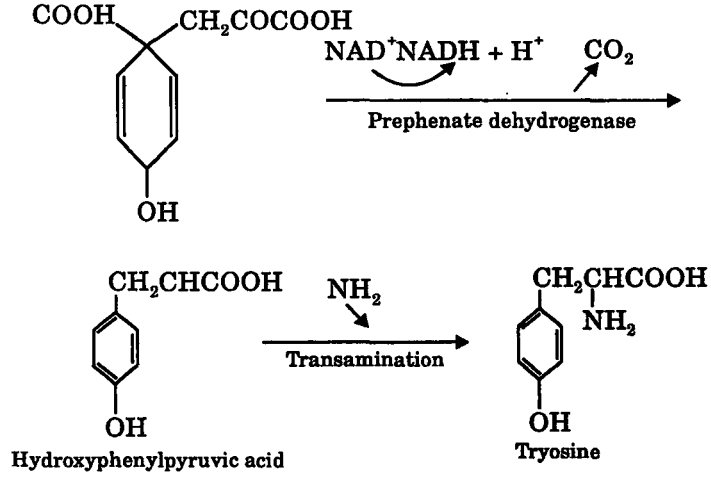
2. Phenylalanine and tyrosine : Chorismic acid gets converted to prephenic acid when there occurs the rearrangements of side groups. This reaction has been catalysed by the enzyme called chorismate mutase.



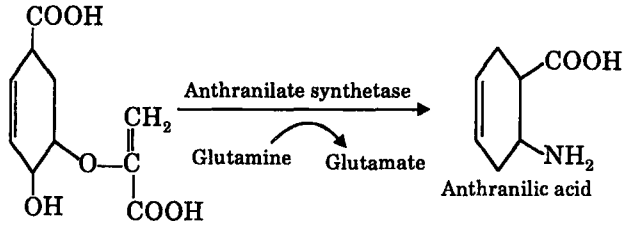
Prephenic acid has been found to be the common precursor for both pheylalanine and tyrosine. In the synthesis of phenylalanine, phenylalanine gets decaboxylated as well as dehydrated to a compound called phenylpyruvic acid which after transmination forms pehylalanine.



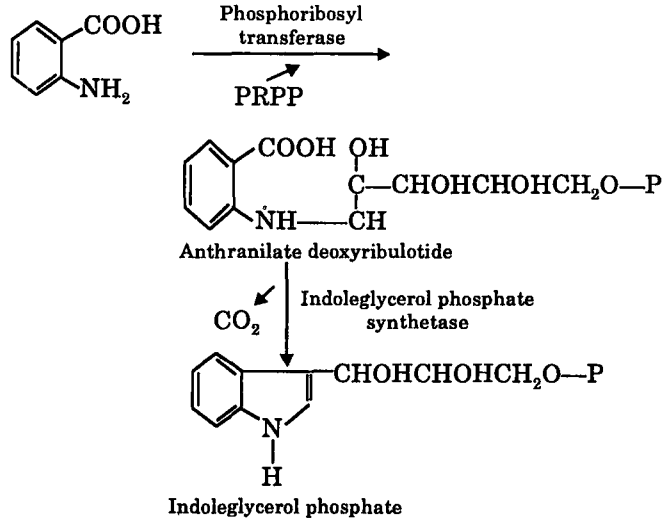
When tryosine is synthesised from prephenic acid, the latter undergoes oxidative decarboxylation first in the presence of enzyme prephenate dehydrogenase to form hydroxyphenyl pyruvic acid which then gets transaminated to yield tyrosine.



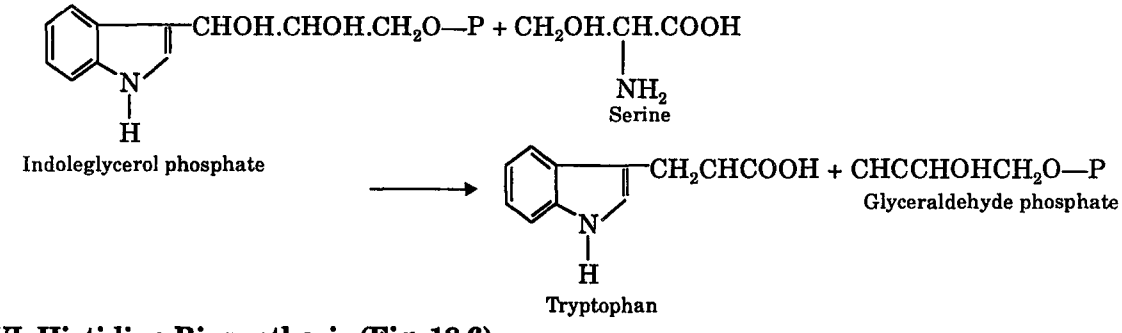
3. Tryptophan : Synthesis of tryptophan is started from chorismate by tits reaction with glutamine when the product of the reaction obtained is anthranilic acid. This reaction takes place in the presence of the enzyme, anthranilate synthetase.



In the next step anthranilic acid undergoes reaction with phosphoribosyl pyrophosphate (PRPP) to form anthranilate deoxyribulotide in the presence of enzyme called anthranilate phosphoribosyl transferrase. In the final step it gets decarboxylated with the formation of side ring also, to form indoleglycerol phosphate.



Synthesis of tryptophan from indoleglycerol phosphate has been reported in *E. coli, Neurospora* and higher plants. The enzyme called tryptophan synthetase is obtained from bacterial and the fungi by crystallisation. Serine undergoes condensation with indole glycerol phosphate in the presence of pyridoxal phosphate to form tryptophan.



VI. Histidine Biosynthesis (Fig. 18.6)

The histidine biosynthesis pathway differs from the biosynthesis pathways of other amino acids in that the carboxyl group is formed in the amino acid during the last reaction. The five carbon atoms in the histidine skeleton are derived from *phosphoribosyl pyrophosphate* (PRPP).

Synthesis of histidine takes place in a series of 10 enzymatic steps which include : (1) Condensation of PRPP and ATP. (2) Cleavage of a pyrophosphate group from the condensation product. (3) Opening of the purine ring. (4) Isomerization of the ribose group originating from PRPP to a ribulose group. (5) and (6) Amido transfer from glutamine, closure of the new imidazole ring and release of the purine nucleotide precursor, aminoimidazole carboxamide ribotide. (7) Dehydration of the glycerol phosphate moiety of imidazole-glycerol phosphate to form *imidazoleacetol phosphate,* an α-keto compound. (8) Transamination reaction. (9) Dephosphorylation. (10) Oxidation of histidinol in two-step reaction to yield histindine.

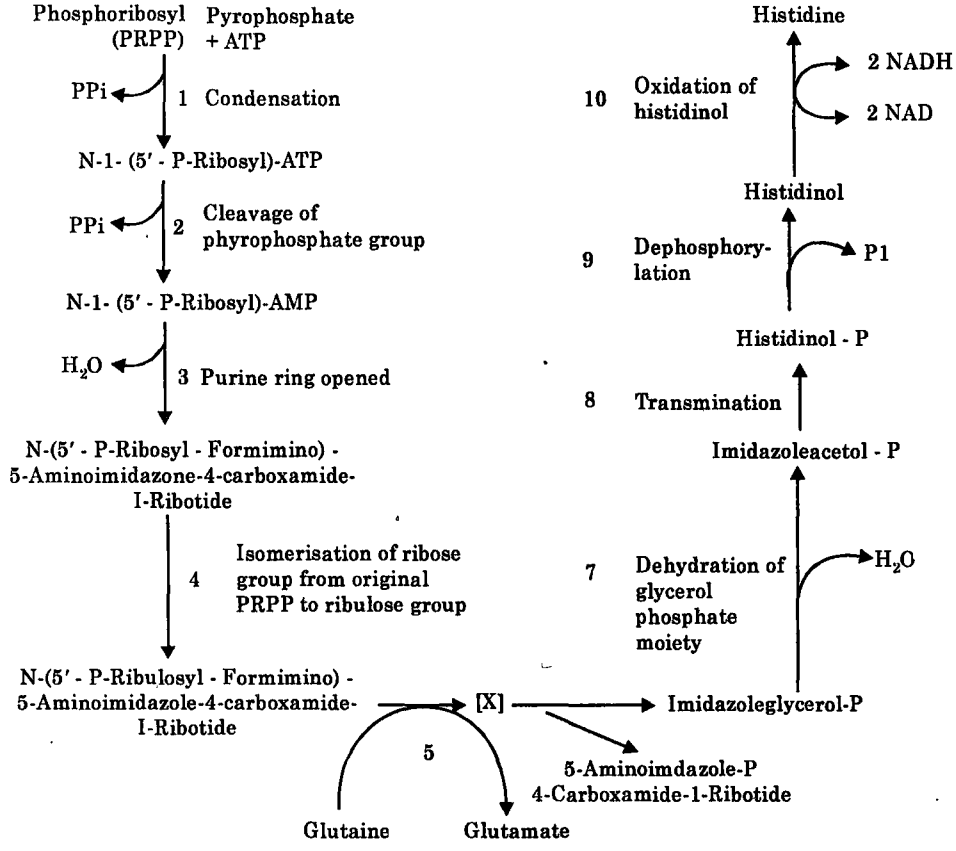


Fig. 18.6. Biosynthesis of histidine.

19 Catabolism of Amino Acids

19.1 Introduction

It is found that many amino acids follow individual metabolic pathways but a few general reactions have been found to be common in the catabolism of nearly all the amino acids. Besides being incorporated into proteins, amino acids also serve as sources of carbon and nitrogen when required. With few exceptions, the first stage in the catabolism of amino acids is the removal of $—NH_2$ group and formation of the corresponding α-keto acids. Ammonia liberated from the amino group is converted quickly into urea or it gets incorporated into some other amino acid.

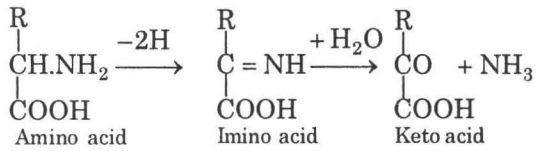
$$R—CH—NH_2COOH \longrightarrow RCOCOOH + NH_3 \longrightarrow \text{Urea}$$

The majority of α-keto acids formed from amino acids join the carbohydrate metabolism whereas a minority are more closely related to the ketone bodies and fatty acids. However, many amino acids are known which do not undergo catabolism in this way but they behave in different individualistic way and are discussed separately.

19.2 Conversion of α-Amino Acid to α-Keto Acids

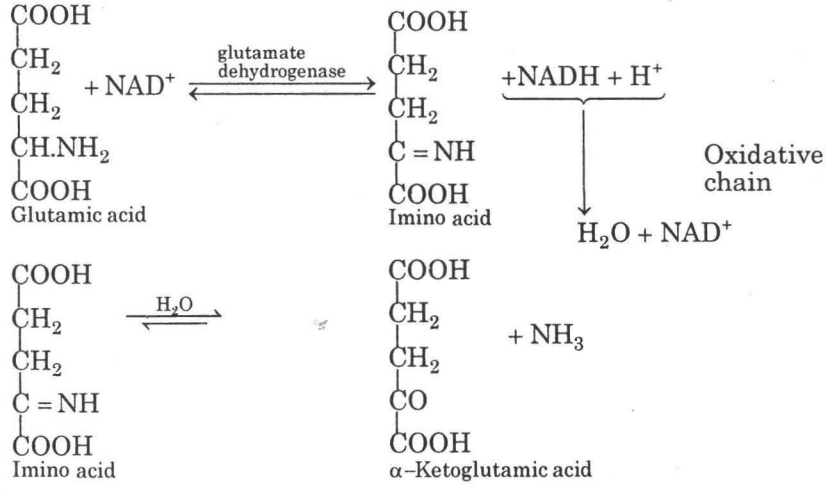
Two important ways are known by which amino acids are converted into their corresponding α-keto acids and thus the amino group has been removed from the carbon skeleton in the form of ammonia.

(i) Oxidative deamination : In oxidative deamination, the amino acids lose two hydrogen atoms to form imino acid which then undergoes hydrolysis to form ammonia and a keto acid.



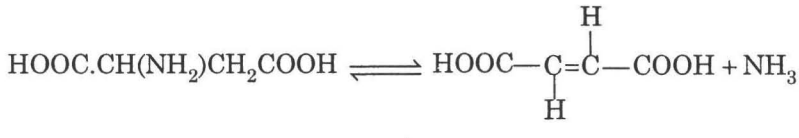
The reaction has been catalysed by the *amino acid oxidase* and the coenzyme (FAD or FMN) which takes up hydrogen. Amino acid oxidases have been found to be of two types depending upon the nature of the substrate on which they are acting. One of these is the *L-amino acid oxidase* which is attacking most of the L-amino acids. The L-amino acid oxidase contains FMN as hydrogen acceptor and occurs mainly in liver and kidney. The other type of amino acid oxidases have been found to be specific for D-amino acids and hence called *D-amino acid oxidases*. These have FAD as the hydrogen acceptor and occur mainly in animal tissues.

Oxidative deamination of glutamic acid may also be catalysed by an important enzyme called the *L-glutamate dehydrogenase*. This enzyme is highly active and occurs abundantly in many tissues. The enzyme is not a flavoprotein and needs NAD as coenzyme. It undertakes the oxidation of glutamic acid to α-ketoglutaric acid (α-oxoglutaric acid).



As the above reaction is reversible, it finds function in both the amino acid catabolism and biosynthesis.

(ii) Non-oxidative deamination : It is catalyzed by amino acid-ammonia lyase. In this process, NH_3 is eliminated by forming a double bond in the remaining carbon-skeleton. In bacteria and plants, aspartate gets deaminated to form fumarate by the enzyme aspartase (aspartate-ammonia-lyase).

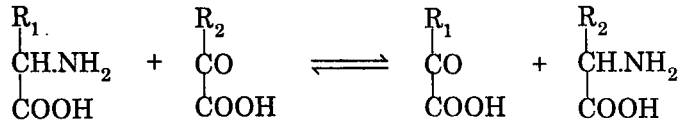


Fumarate

Similarly, phenylalanine can be deaminated to form cinnamate. Deamination of histidine occurs more common in mammals.

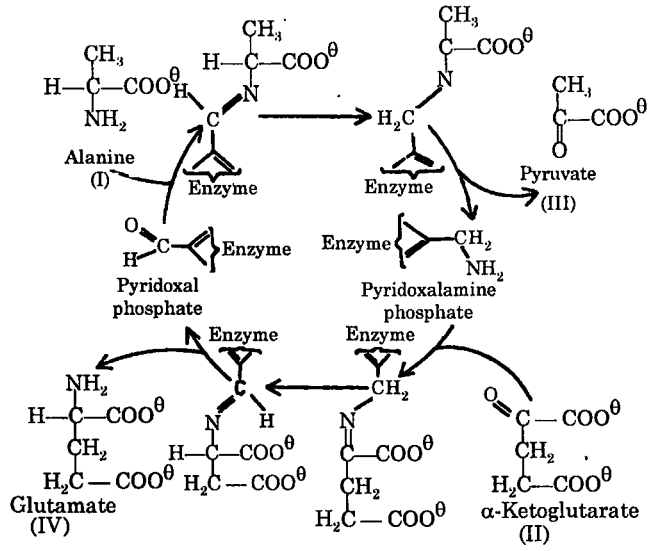
(iii) Transmination : It is the most important mechanism involving the conversion of an amino acid into keto acid. Its importance was revealed for the first time by Russian workers Braunstein and Bychkou in 1939.

Transamination involves the transference of an amino group from a donor amino acid to a recipient keto acid in the presence of enzyme *transminase or aminotransferase.*



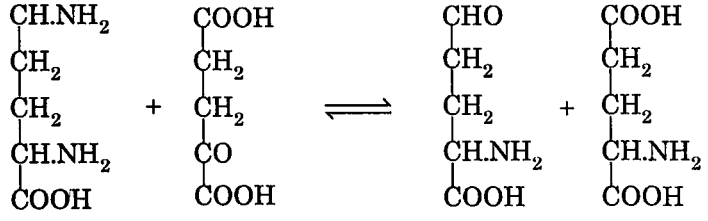
I II III IV

The donor amino acid is converted into a keto acid while recipient keto acid is converted into an amino acid, the coenzyme required for this reaction is *pyridoxal phosphate.*



Pyridoxal phosphate reacts with the amino acid to form a Schiff's base type of complex which then gives the keto acid and pyridoxalamine phosphate. The latter now reacts with a second keto acid to form a Schiff's base complex which again decomposes to form an amino acid and *pyridoxal phosphate.*

Amino acids (but not all) except lysine, threonine and the cyclic amino acids, proline and hydroxyproline undergo transamination. Moreover, transaminations involving the β, γ, or δ-amino acids, aldehydo-acids, and even D-amino acids (in bacteria) have been also reported.



L-Ornithine (a δ-amino acid) | α-Ketoglutaric acid | L-Glutamate γ-semialdehyde | Glutamic acid

As L-glutamate is the only amino acid in mammalian tissues which can undergo oxidative deamination due to the presence of highly active enzyme called *L-glutamate dehydrogenase*, all other amino acids get converted to glutamic acid by transamination with α-ketoglutaric acid. The glutamic acid then gets oxidative deaminated to form α-ketoglutaric acid and ammonia.

The process of amino acid catabolism involving the combined action of an amino, transferase (transaminase) and glutamate dehydrogenase may be depicted in Fig. 19.1.

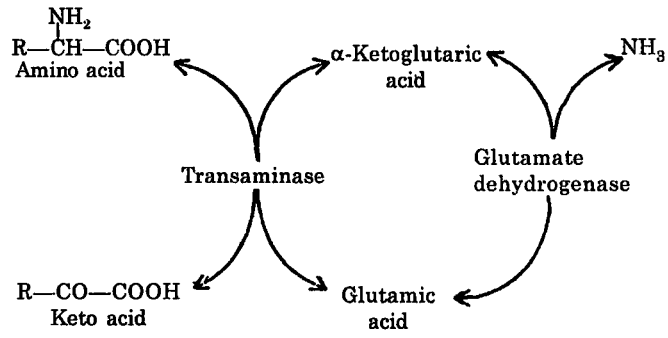
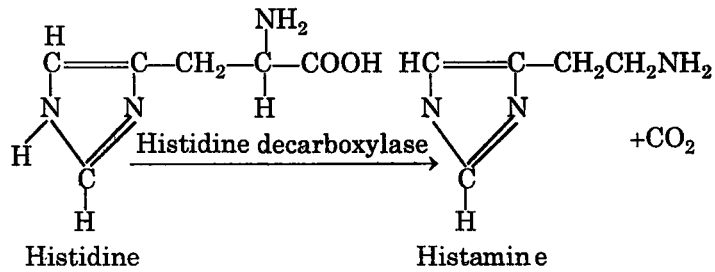


Fig. 19.1. Overall catabolism (transdeamination) of amino acids.

19.3 Decarboxylation of Amino Acids

Amino acid decarboxylases have been found to catalyse the removal of CO_2 from the carboxyl group of amino acids. All decarboxylases need pyridoxal phosphate as coenzyme. For example, histidine has been decarboxylated to histamine by histidine decarboxylase.



Also, 3, 4-dihydroxyphenylalanine has been decarboxylated to dopamine, tryptophan to tryptamine, tyrosine to tyramine, glutamate to amino butyrate, and so on. Such amines are called biogenic amines. Many of these amines possess strong pharmacological effects while others are important as precursors of hormones or as coenzymes.

The various amino acids and their corresponding biogenic amines with their significance are given is Table 19.1.

Table 19.1
BIOGENIC AMINES

Amino acid	*Decarboxylation product*	*Significance*
1	*2*	*3*
Serine	Ethanolamine	Phosphatides
Threonine	Propanolamine	Vitamin B_{12}
Cysteine	β-Mercaptoethyl amine	Coenzyme A
Aspartic acid	β-Alanine	Coenzyme A, Pantothenic acid
Glutamic acid	γ-Aminobutyric acid	Brain (ganglia inhibitor)

1	*2*	*3*
Histidine	Histamine	Vasodilator and hence decreases blood pressure
Tyrosine	Tyramine	Uterus contracting, increases blood pressure
3, 4-Dihydroxy-phenylalanine	Dopamine (→ epinephrine)	Tissue hormone (→ hormone)
Tryptophan	Tryptamine	Hormone
6 Hydroxy-tryptophan	Serotonin (→ Melatonin)	Tissue hormone (hormone)

Except the decarboxylation of glutamic acid in brain tissue, other decarboxylation reactions have been found to be irreversible.

19.4 Disposal of the Nitrogen

Ammonia is constantly produced in the tissues. It is a toxic substance and should be removed rapidly from the circulation (*detoxication*) by the liver which converts it (ammonia) into glutamate, glutamine or urea. The various paths for the fixation of ammonia obtained from amino acids are as follows :

(i) Synthetic pathways.

(ii) Gutamate pathway.

(iii) Formation of urea.

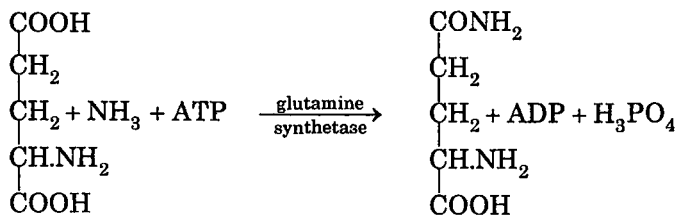
(iv) Direct excretion.

(v) Formation of creatine and creatinine.

We shall discuss these one by one.

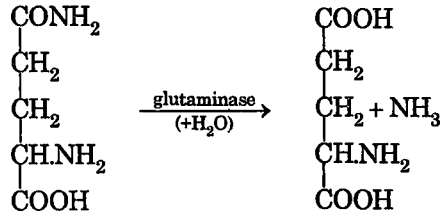
(i) Synthetic pathways : Ammonia may involve in the reductive amination of α-keto acids (derived from carbohydrates) to form new amino acids. This is termed as reversal of transdeamination reactions. Ammonia may also involve in the synthesis of purines, pyridines and porphyrins. In these syntheses, ammonia does not act in the free state but in the form of carrier such as glutataminate, aspartate, carbamoyl-phosphate and glycine.

(ii) Glutamate pathways : In extrarenal tissues ammonia is converted into glutamine in the presence of glutamine synthetase, an enzyme present primarily in the brain and liver.



L-Glutamic acid L-Glutamine

Glutamine is not a toxic substance and it travels from the various tissues through the blood to the kidneys where it gets hydrolysed by *glutaminase* to glutamic acid and ammonia.

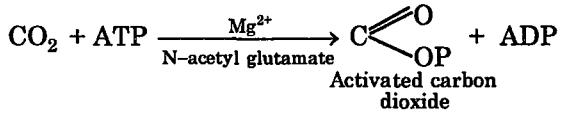


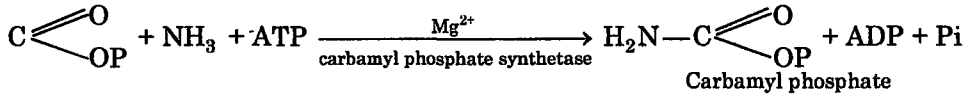
L-Glutamine L-Glutamic acid

The ammonia thus liberated accounts for about 60% of the urine ammonia.

(iii) Direct excretion : Usually deamination of amino acids takes place in extrarenal tissues in which the ammonia is immediately channelled into certain metabolic pathways which bind it. If the removal of amino group from the amino acid (deamination) takes place in kidney in the absence of immediate physiological requirements for synthetic purposes, the liberated ammonia gets excreted directly into the urine. The source of urinary ammonia accounts for about 40% of the total urinary ammonia (60% of urinary ammonia is obtained by the hydrolysis of glutamine in kidney).

(iv) Formation of urea : The conversion of ammonia to urea in the liver occurs by the ornithine cycle proposed by Krebs. This cycle starts with a carrier molecule called ornithine (amino acid). Before the actual ornithine cycle takes place, ammonia (derived by deamination of amino acids) and carbon dioxide (derived from Krebs cycle) combine in the presence of ATP to form carbamyl phosphate (amido phosphate). This reaction takes place in two steps :





After the formation of carbamyl phosphate the actual ornithine cycle starts in which the former compound undergoes reaction with the α-amino group of ornithine to form citrulline in the presence of *ornithine carbamyl transferase.*

Citrulline then undergoes condensation with the amino group of aspartate to form arginosuccinate. The reaction needs ATP and has been catalysed by the *arginosuccinate synthetase.* Arginosuccinate gets cleaved reversibly to a mixture of fumarate and arginine by the enzyme *arginosuccinase.* Finally, arginine gets cleaved by the enzyme *arginase* into urea and ornithine. This has completed the cycle. Now ornithine molecule accepts another molecule of carbamyl phosphate to repeat this cycle.

In summary, two ammonia (from glutamate and asparate) and one CO_2 gives urea. In the process, 3 moles of ATP are consumed.

Ornithine cycle of urea synthesis is shown in Fig. 19.2.

(v) Creatine and creatinine : Creatinine is the anhydride of creatine. Its constant amount (related to muscle mass) is excreted daily. Formation of these have been described elsewhere in this chapter because these are formed only from three amino acids, namely, glycine, arginine

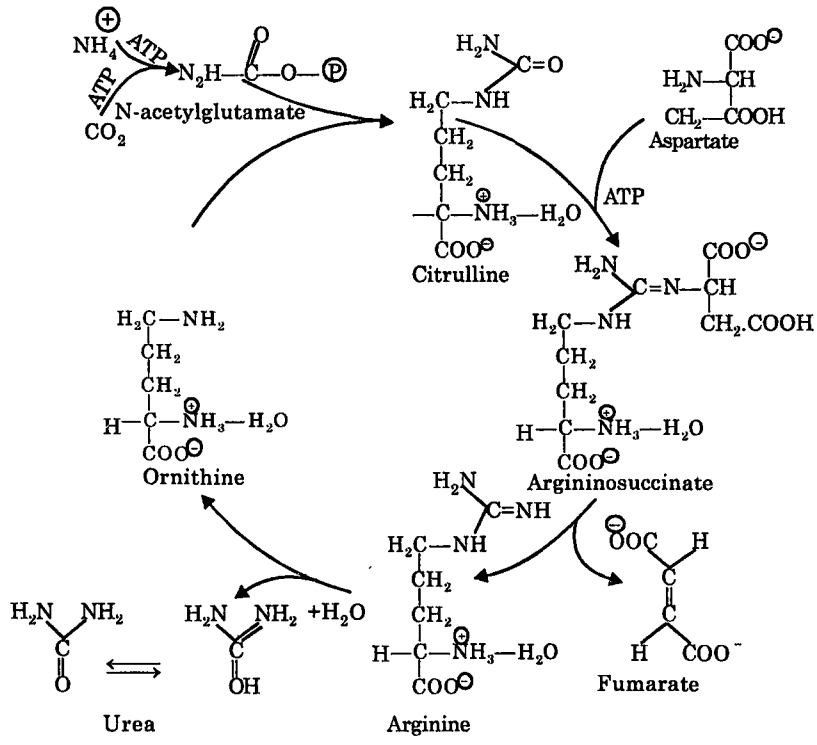
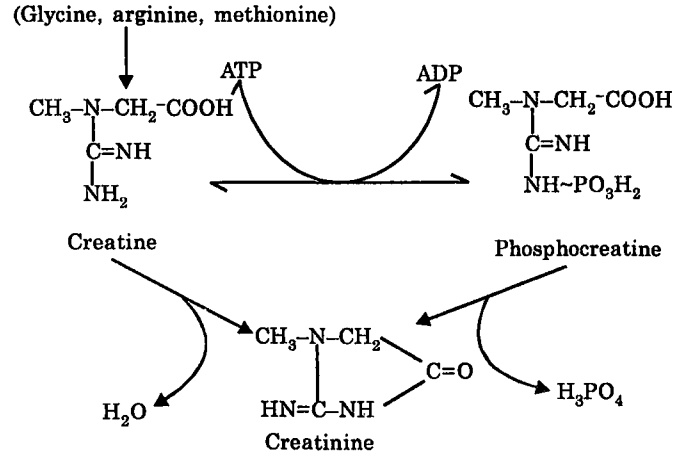


Fig. 19.2. Urea synthesis by the ornithine cycle.

and methionine rather the entire group of amino acids, However, the relationship of these compounds to each other has been depicted as follows :



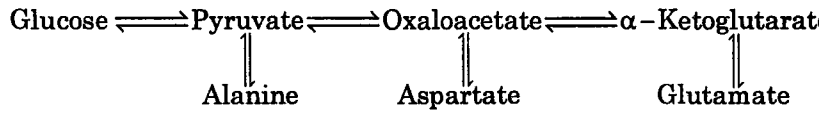
19.5 Disposal of Carbon Skeleton

The fate of keto acids, which are obtained by the removal of amino group as ammonia, may be either of the following types :

(i) Synthetic pathways : The α-keto acids which are formed by deamination may also undergo amination reductively by reversing transdeamination mechanism, thus reforming the original amino acids. This process is continuous.

(ii) Glucogenic pathway : The carbon skeleton of most of the amino acids may be converted into carbohydrates (gluconeogenesis from protein). Such amino acids are termed as *glucogenic*

or antiketogenic amino acids. A few amino acids involving in carbohydrate metabolism directly, are depicted below :



The routes for the conversion of various keto acids to carbohydrates have been found to depend upon the compound concerned (for details see metabolism of individual amino acids). The various glucogenic amino acids are given in Table 19.2.

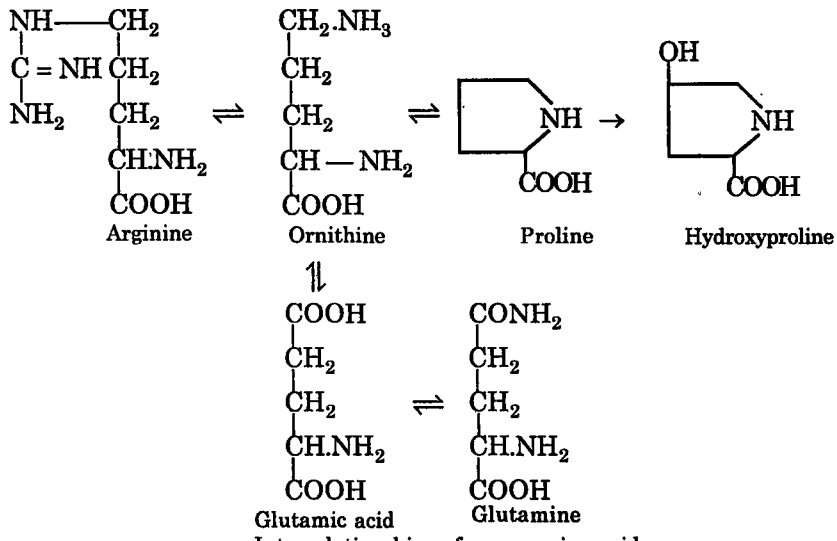
The keto acids derived from these amino acids may also directly enter the tricarboxylic acid cycle and thus get oxidised ultimately to CO_2 and H_2O. For example, pyruvic acid obtained by the deamination of alanine gets oxidised to CO_2 and H_2O *via* TCA cycle.

Table 19.2

AMINO ACIDS ACCORDING TO THE FATE OF THEIR CARBON SKELETON

Glycogen forming (Glycogenic amino acid)	*Fat forming (Ketogenic amino acid)*	*Both glycogen and fat forming (Glycogenic as well as ketogenic amino acids)*
Alanine Hydroxyproline	Leucine	Isoleucine
Arginine Methionine		Lysine
Asparate Proline		Phenylalanine
Cystine Serine		Tyrosine
Glutamic acid Threonine		Tryptophan
Glycine Valine		
Histidine		

(iii) Ketogenic pathway : The α-keto acid (isovaleryl formic acid) derived from the deamination of leucine, on its oxidation to CO_2 and H_2O has to pass through the stage of acetoacetic acid and thus ketone bodies instead of glucose are formed. Such amino acids are termed as *ketogenic amino acids.*



Interrelationships of some amino acids

(iv) Glucogenic as well as ketogenic pathway : The keto acids derived from certain amino acids may enter the above-mentioned glucogenic as well as ketogenic pathways, thereby forming both glucose and ketone bodies. Such amino acids are isoleucine, lysine, phenylalanine, tyrosine and tryptophan.

(v) Other pathways : The metabolic pathways of certain amino acids do not correspond with either of the above pathways. These routes are highly specific and have been described in the appropriate sections.

Interrelationships of amino acids : Isotopic experiments show that arginine, ornithine, proline and glutamic acid could be interconverted by keeping the carbon chain intact.

19.6 Catabolism of Individual Amino Acids

The products obtained by the degradation of various amino acids ultimately enter the tricarboxylic acid cycle. The nature of these intermediates ascertains the nature of amino acids, *i.e.*, glucogenic, ketogenic or both. On the basis of the intermediates formed in the degradation, the various amino acids have been classified into the following five groups.

(*i*) *Amino-acids forming fumarate, e.g.,* Aspartate and asparagine, tyrosine, and phenylalanine.

(*ii*) *Amino-acids forming glutamate and hence α-ketoglutarate, e.g.,* Glutamic acid, glutamine, proline, arginine, and histidine.

(*iii*) *Amino-acids, forming pyruvate, e.g.,* Alanine, serine, cysteine, cystine, threonine, glycine, and hydroxyproline.

(*iv*) *Amino-acids forming acetyl—CoA, e.g.,* Phenylalanine tyrosine, lysine, tryptophan and leucine.

(*v*) *Amino-acids forming succinyl—CoA, e.g.,* Methionine, leucine, valine and isoleucine.

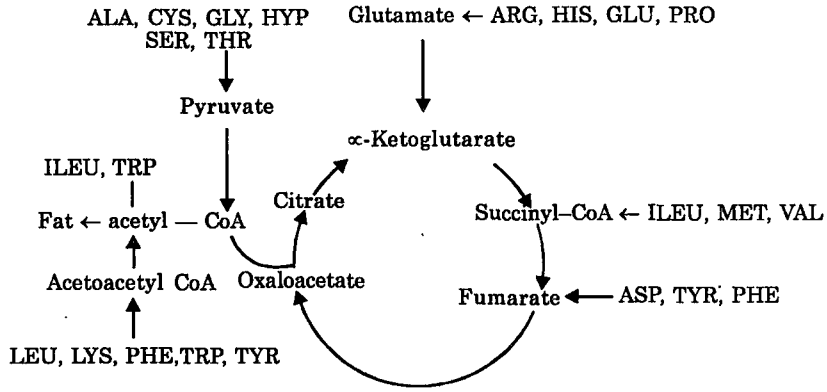


Fig. 19.3. Intermediates from various amino acids.

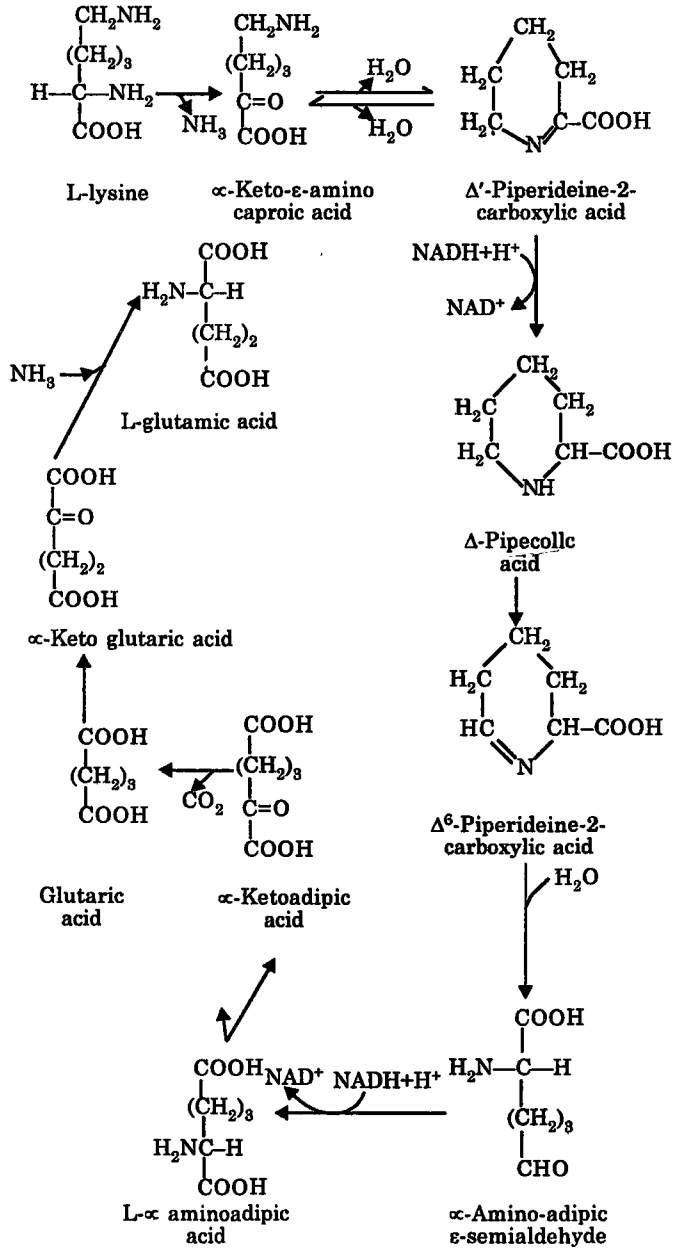
It will be evident from the catabolism of individual amino acids, all the 13 glycogenic amino-acids are ultimately converted to oxaloacetate. The latter is then converted to glycogen via phosphoenol pyruvic acid (see gluconeogenesis). On the other hand, the ketogenic amino acids get degraded to acetyl—CoA (some *via* acetoacetyl-CoA, *viz.*, leucine, lysine, pheylalanine, tryptophan and tyrosine) which may be changed to fat as well as to glycogen. The conversion of the intermediates of the various amino-acids into oxaloacetate is shown in Fig. 19.3.

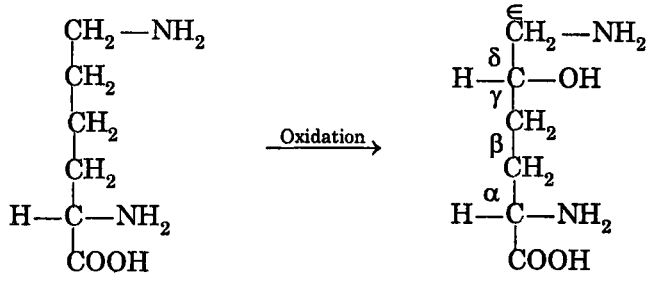
1. Lysine : It is an essential amino acid which is required in the diet for growth of maintenance of nitrogen balance. D-Isomer of lysine cannot be utilised.

Lysine gets deaminated by an irreversible process to form α-ketoglutaric acid. This has been studied by isotopes and by other studies, is very peculiar and is sketched below. It reveals that lysine is potentially glucogenic. However, it is quite difficult to demonstrate by the usual methods.

Hydroxylysine : Hydroxylysine, a derivative of lysine, is synthesised from lysine by oxidation in the δ-position.

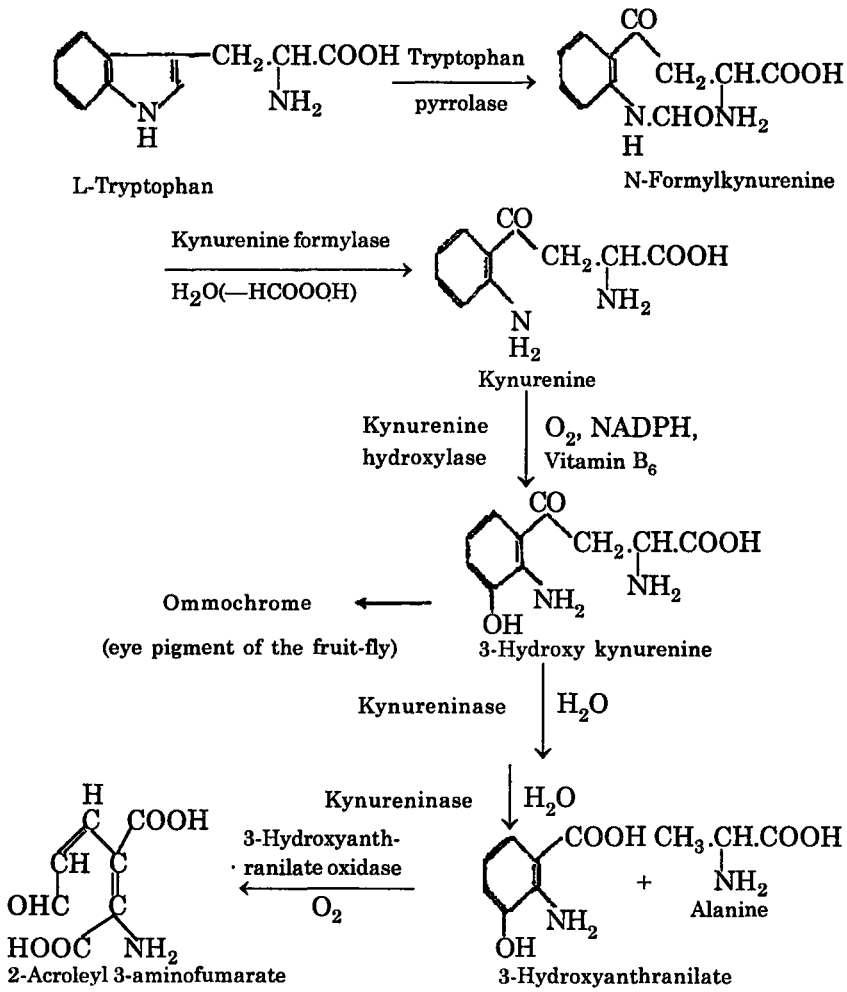
Hydroxylysine occurs in the collagen, gelatin and also in the enzyme protein trypsin and chymotrypsin. It is accepted that lysine gets converted to hydroxylysine during its incorporation into protein.

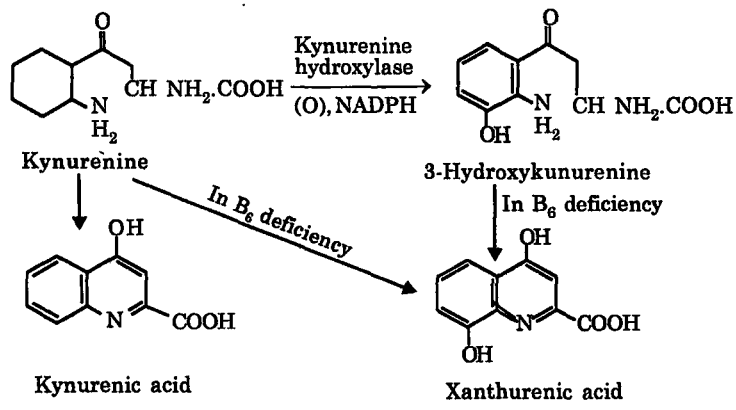




2. Tryptophan : It is an essential amino acid. It is the only amino acid having the indole ring. Its metabolism takes place through several pathways which are found in different species of animals. Some of these pathways are described as follows :

(A) Kynurenine or nicotinamide pathway : It is the main pathway for the metabolic breakdown of tryptophan. By this pathway, it gets converted into nicotinic acid (a vitamin), $NAD^+ + NADP^+$

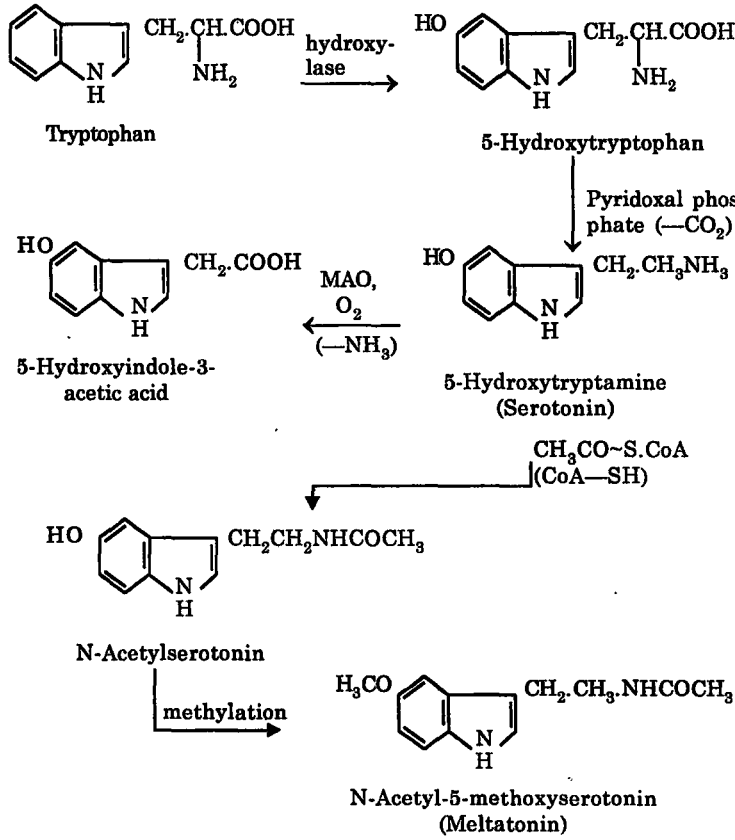




Conversion of kynurenine and hydroxykynurenine

2-Acroleyl 3-aminofumarate is very unstable. It may undergo catabolism mainly in two important ways to form different products.

(a) It may get cyclised to the pyridine derivative *quinolinate* which gets converted enzymatically to its ribonucleotide by reaction with phosphoribosyl pyrophosphate. The same enzyme then brings about decarboxylation to form nicotinate ribonucleotide (mononucleotide), which becomes a dinucleotide with ATP. Finally, glutamine donates an NH_2 group thereby forming nicotinamide adenine dinucleotide (NAD^+).



(*b*) The above described route of 2-acroleyl 3-aminofumarate (and thus tryptophan) yielding the formation of the coenzymes NAD^+ and $NADP^+$ is a minor pathway. In the main pathway, the above unsaturated aldehyde gets decarboxylated to form 2-aminomuconate 6-semialdehyde which may undergo cyclisation to form picolinate or dehydrogenation at the aldehyde group to form α-aminomuconate. The aminomuconate is a labile enamine and is hydrolysed readily to form ammonia and oxalocrotonate. Hydrogenation of the double bond of the oxalocrotonate with NADH gives α-ketoadipate, which undergoes oxidative decarboxylation thereby forming glutaryl coenzyme A.

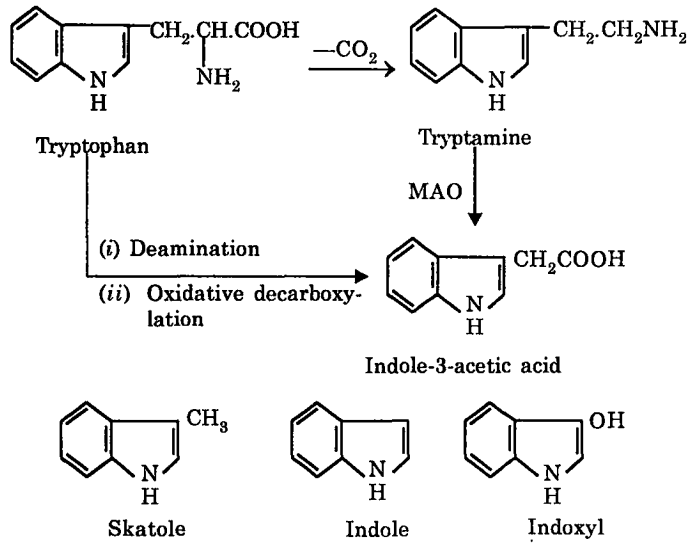
(B) Serotonin pathway : It is a secondary pathway for the metabolism of tryptophan, which involves its hydroxylation to 5-hydroxytryptophan. Decarboxylation of 5-hydroxytryptophan yields 5-*hydroxytryptamine* (5. HT), also known as *serotonin, enteramine,* or thrombocytin.

Serotonin is formed widely in both animals and plants. It occurs in the blood, most cells and blood platelets.

(C) Other pathways : Under certain conditions as are found in the colon, tryptophan may yield the evilsmelling compounds, *viz., skatole and indole* and the related substance *indoxyl* which may come in the feces and urine. Indole-3-acetic acid formed by decarboxylation and oxidation of tryptophan or indole pyruvic acid also appears in urine.

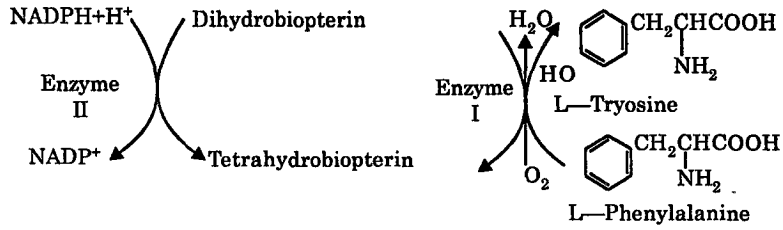
3. Phenylalanine and tyrosine (Aromatic amino acids) : The metabolism of the two aromatic acids yields compounds of great physiological importance, *viz.,* thyroxine, adrenaline, melanine, etc.

The catabolism of phenylalanine starts with its irreversible conversion to tyrosine which occurs in the liver. This reaction takes place in the following steps :



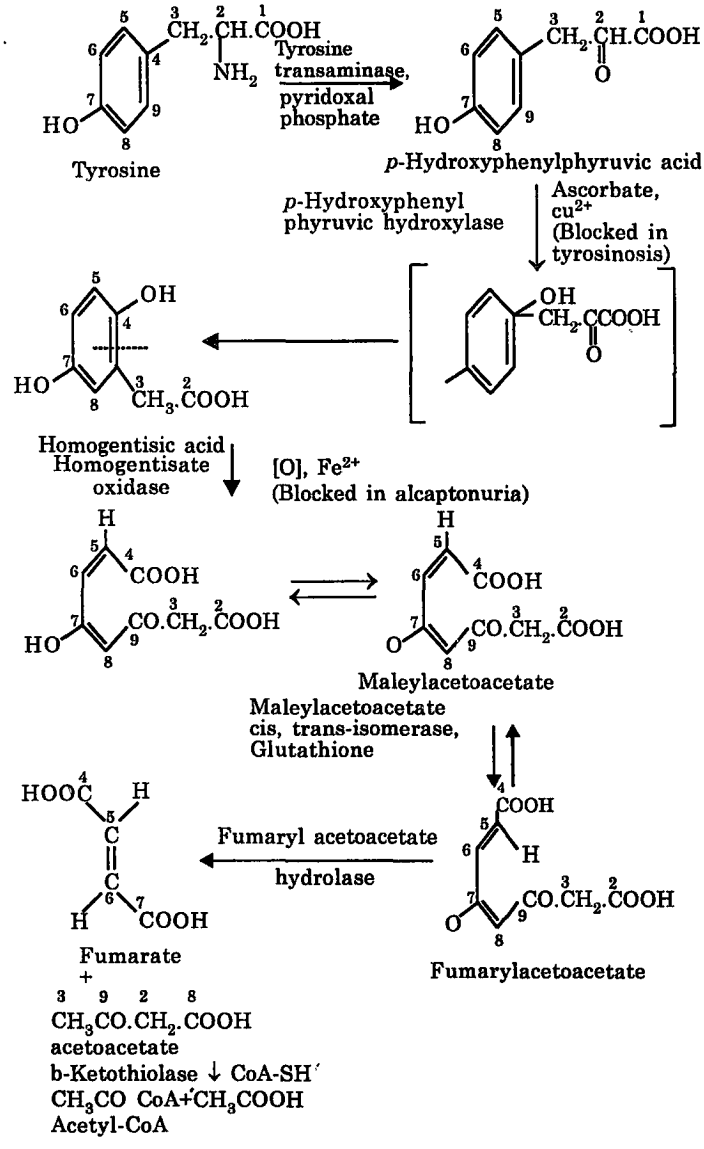
(*a*) In the first step, the oxidation of phenylalanine to tyrosine is catalysed by a specific hydroxylase, enzyme I (phenylalanine-4-hydroxylase) with a reduced form of the pteridine cofactor acting as hydrogen donor to the molecular oxygen.

(*b*) In the second step, the oxidised pteridine cofactor gets reduced by NADPH in the presence of enzyme II.



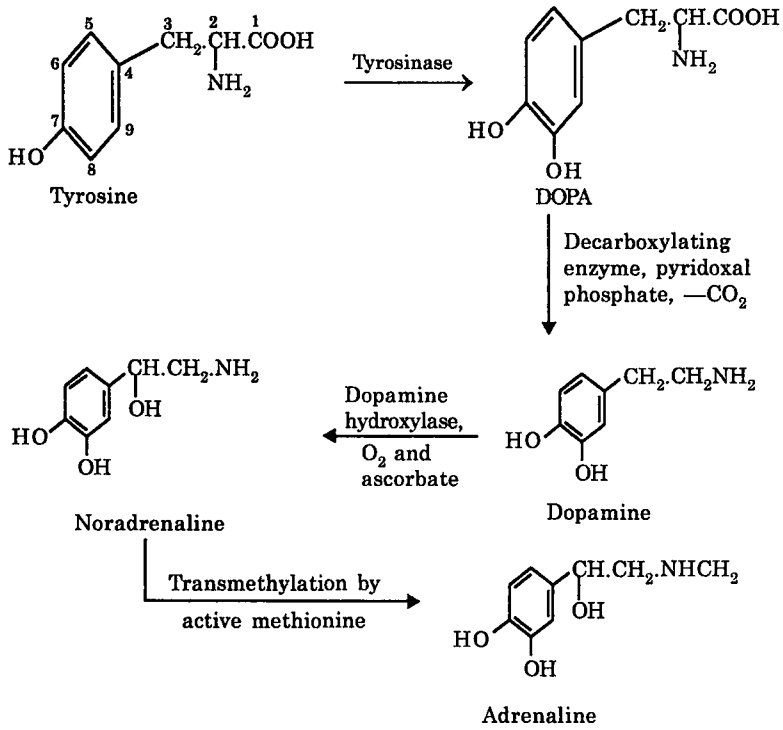
Conversion of phenylalanine to tyrosine

Tyrosine formed from phenylalanine or arising directly from ingested proteins has various routes available.

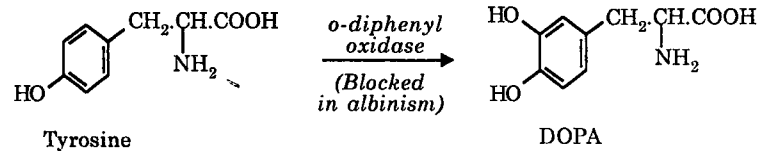


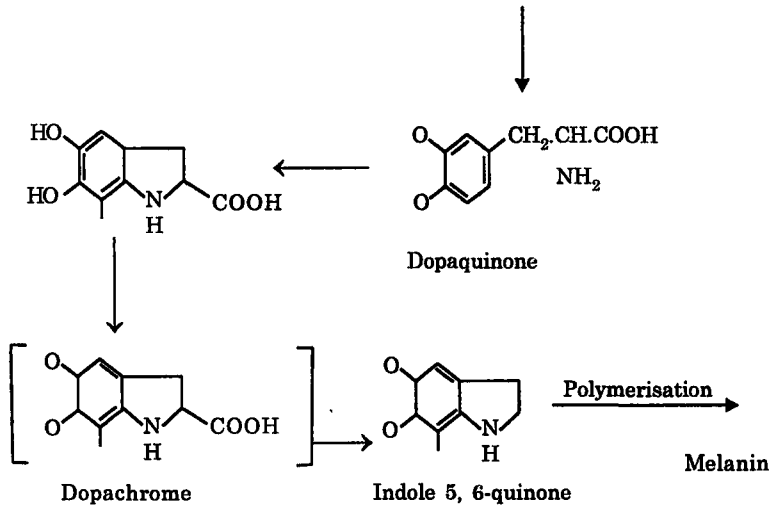
(a) Acetoacetate and fumarate pathway : Most of the phenylalanine and tyrosine undergo catabolism daily *via* this route.

(b) Epinephrine (adrenaline) pathway : Tyrosine is first oxidised to form 3, 4-dihydroxyphenylalanine (DOPA) in the presence of a specific hydroxylase. DOPA gets subsequently decarboxylated by an active decarboxylating enzyme to hydroxytyramine or dopamine. Pyridoxal phosphate is the coenzyme in this step. The next step involves the hydroxylation of the proximal carbon atom in the side chain of dopamine to form norepinephrine (noradrenaline). This reaction has been catalysed by dopamine hydroxylase, which needs molecular oxygen and ascorbate. Epinephrine (adrenaline) gets synthesised from norepinephrine by transmethylation, S-adenosyl-methionine being the source of the methyl group.

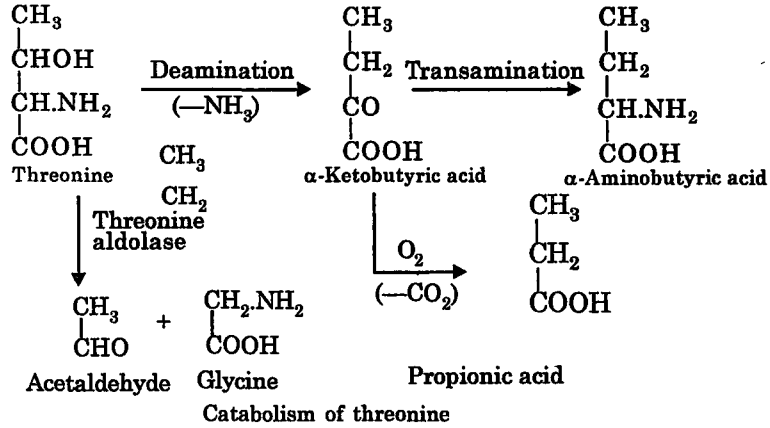


(c) Pathway to melanin : The whole sequence of reactions leading to the formation of melanin from oxidation products of DOPA is catalyzed by a single enzyme, *o-diphenol oxidase* (formerly known as *catechol oxidase or tyrosinase*). The enzyme converts tyrosine to DOPA (dihydroxyphenylalanine) which gets oxidised further to the corresponding quinone derivative. The quinone derivative then gets converted to melanin through a series of reactions.



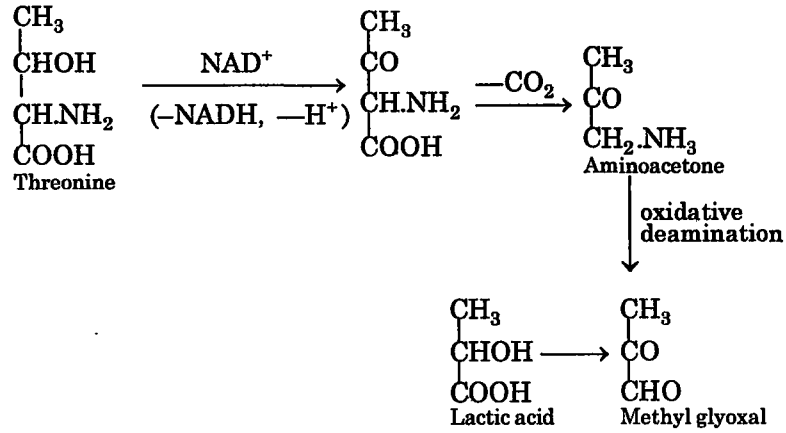


4. Threonine : It is one of the few amino acids which do not involve in transamination. Its breakdown is related to glycine.

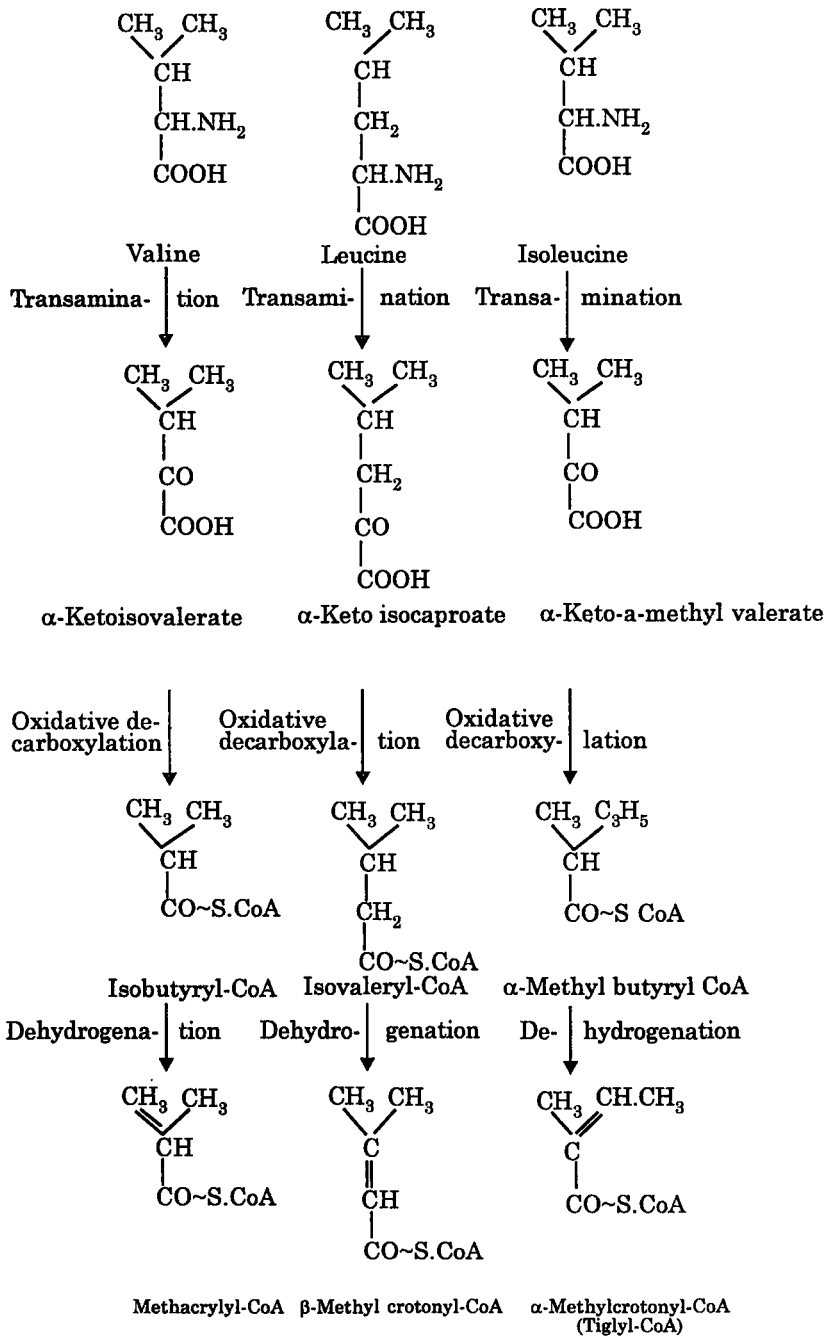


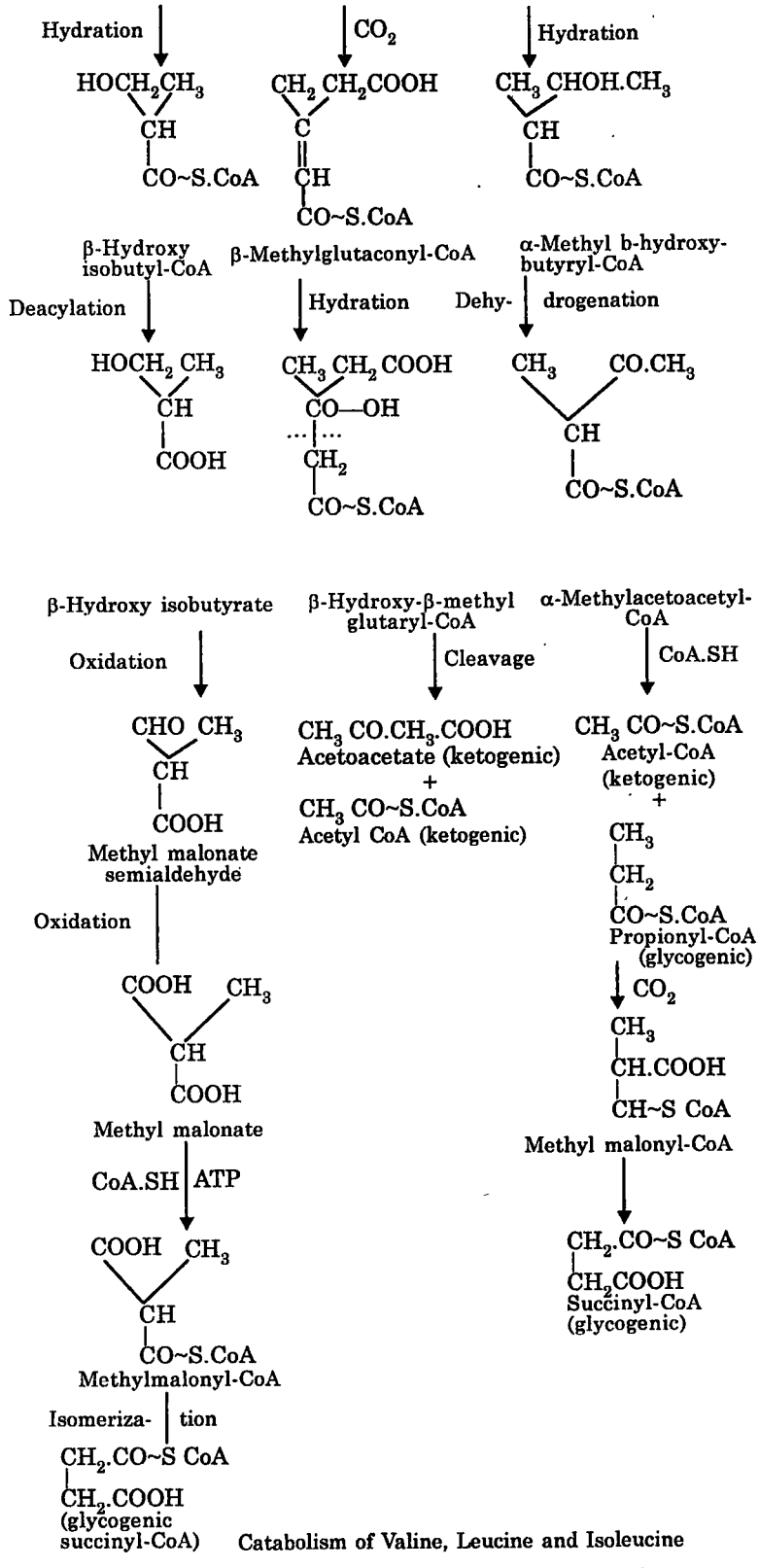
Catabolism of threonine

Other catabolic pathway of threonine first forms the amino acetone and then methylglyoxyal and lactate as shown below :



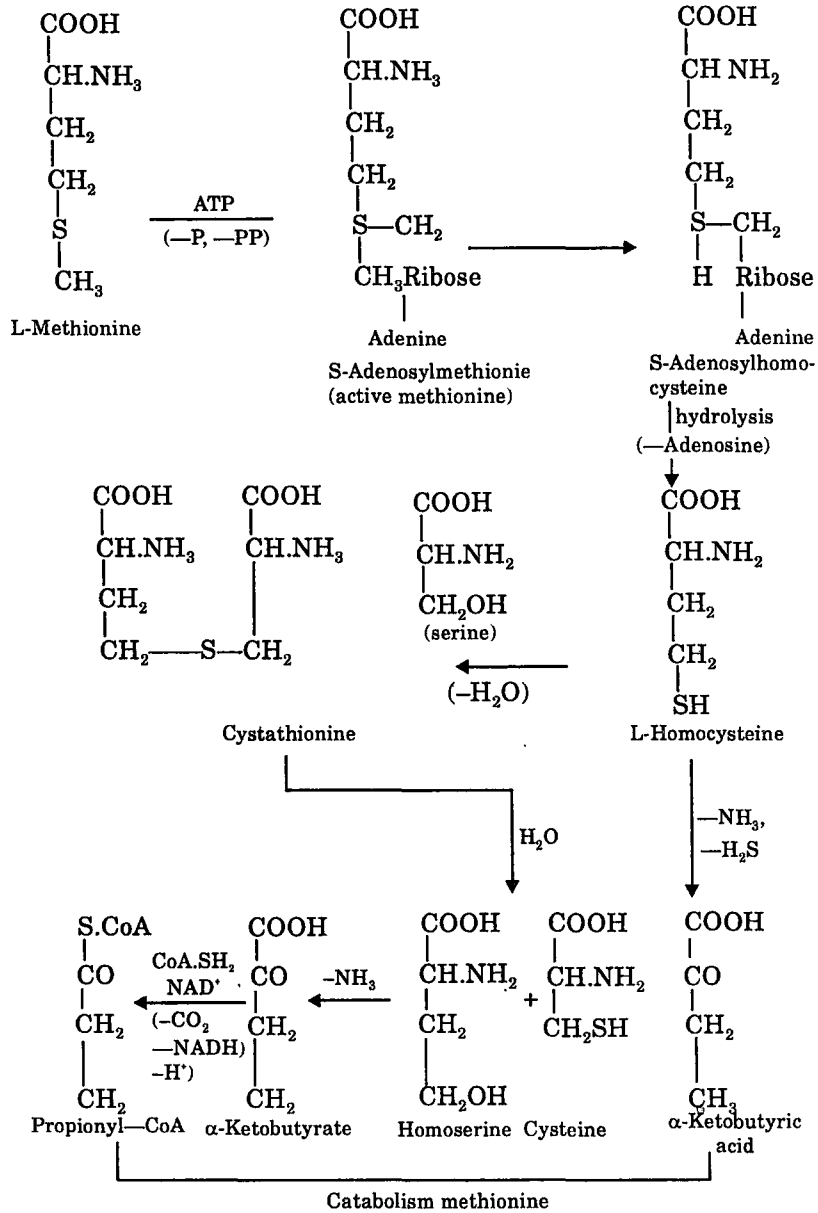
5. Valine, leucine and isoleucine : These are branched chain amino acids which are degraded in a quite analogous manner. The first three steps in the catabolism of these amino acids involve the same reactions after which the reactions of the three products (α, β-unsaturated acyl—CoA thioesters) follows different paths, to form products. The nature of these products has been found to depend upon the fact whether each amino acids is glycogenic (valine) ketogenic (leucine) or both (isoleucine).





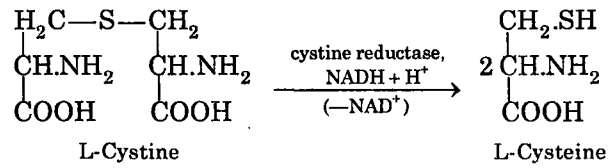
Catabolism of Valine, Leucine and Isoleucine

6. Methionine : It is an important amino acid. The first step in the catabolism of methionine involves its activation with ATP to form S-adenosylmethionine (active methionine). Then it proceeds in the following ways :



SEMIDISPENSABLE AMINO ACIDS

7. Cyteine and cystine : The conversion of methionine to cysteine is described in the catabolism of methionine whereas the conversion of cystine to cysteine is effected in the presence of an NADH— dependent oxidoreductase.

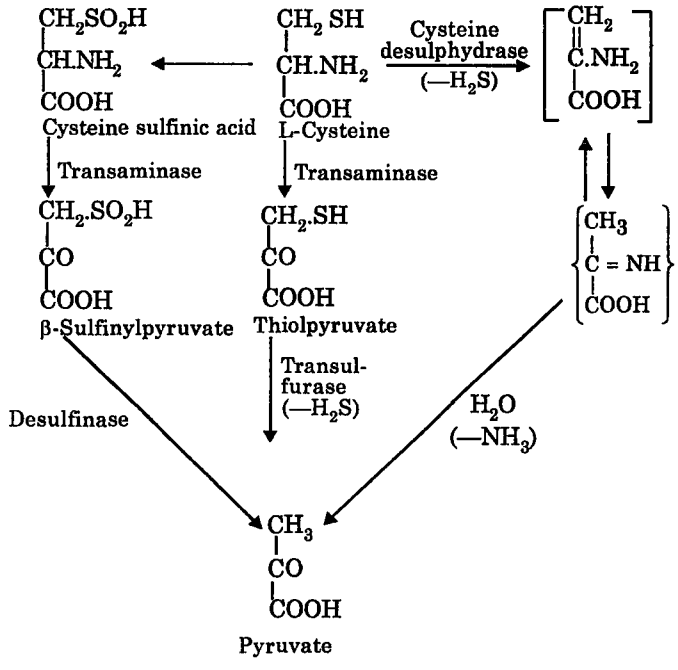


Cysteine is mainly degraded to pyruvic acid. However, other metabolic products of cysteine are glutathione, taurine, β-mercaptoethylamine (a constituent of coenzyme A), and mercapturic acid. Cysteine also gets involved in the synthesis of insulin.

Pyruvate Pathways : Cysteine may get degraded to pyruvate in three different ways :

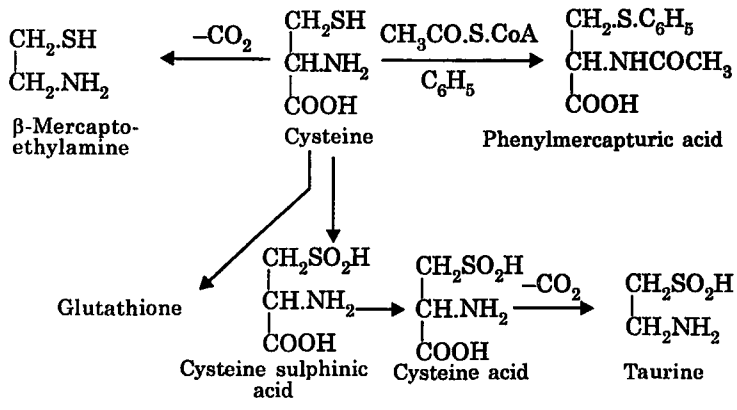
(*a*) It may get degraded by the cysteine desulphydrase to pyruvate, H_2S and NH_3.

(*b*) Cysteine may also get transaminated with an a-keto acid to form thiolpyruvate which on decomposition yields pyruvate and H_2S. The latter step gets catalysed by transulfurase.

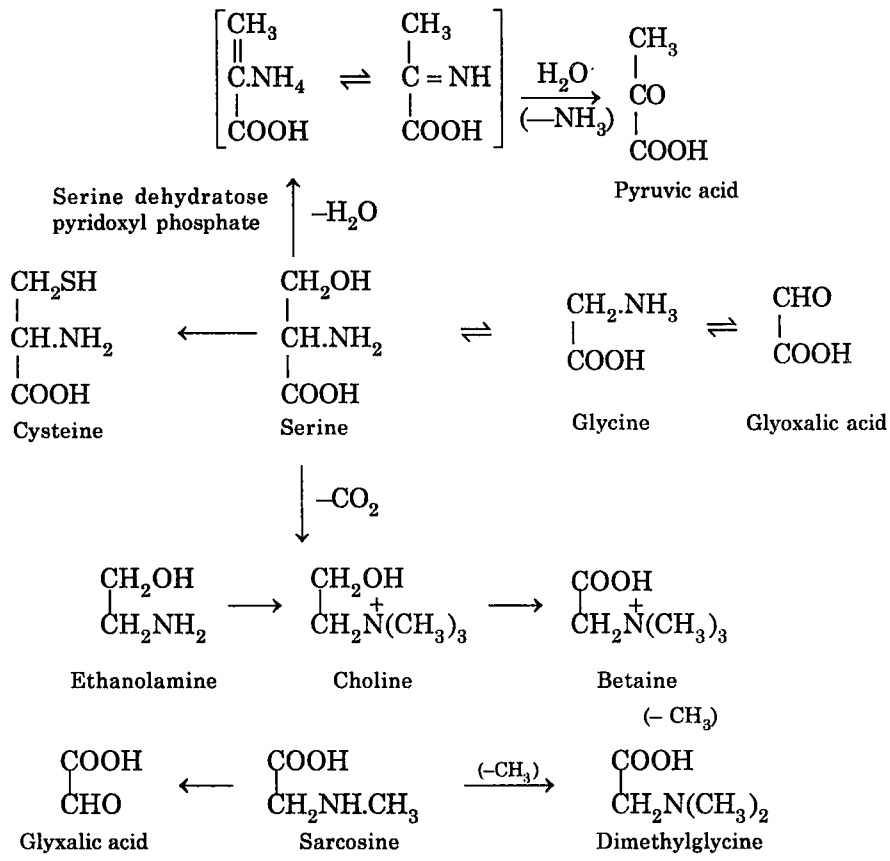


(*c*) Cysteine may also get covered to pyruvate by oxidation of the sulfhydryl group to form cysteine sulfinic acid, followed by transamination and loss of the oxidised sulphur acid.

Formation of the Other Products : The cysteine, in addition to pyruvate, also gets converted to various other products, *viz.*, taurine on combination with cholic acid forms the bile acid (taurocholic acid). β-mercaptoethylamine, mercapturic acid, and glutathione. A summary of these degradations of cysteine may be given as follows :

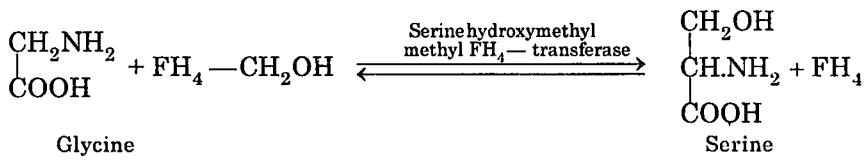


8. Serine : It is non-essential amino acid. Its various metabolic paths are as follows :



9. *Glycine :* It is a glucogenic non-essential amino acid which can be synthesised from threonine and from serine. However, its degradation may take several pathway given below :

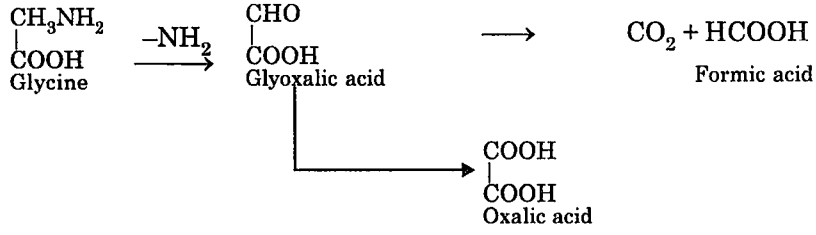
(*i*) One of the degradative pathways of glycine is simply the reverse of its synthetic pathway in serine.



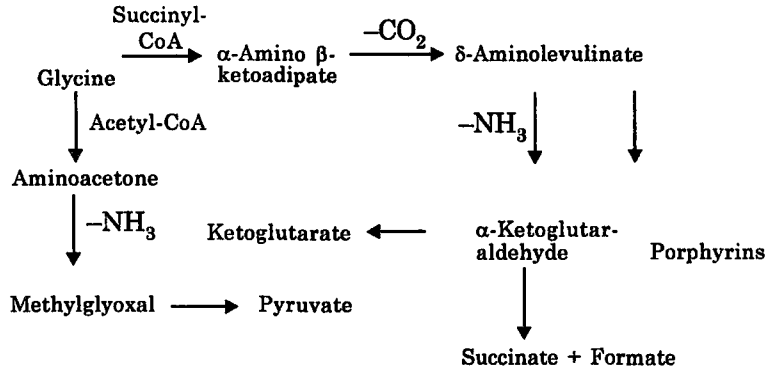
Where FH_4 = Tetrahydrofolic acid

Now serine gets degraded to pyruvic acid which is glycogenic in nature.

(*ii*) Glycine may get deaminated mainly by transamination and to a smaller extent by glycine oxidase to glyoxalate which may get degraded by oxidative decarboxylation to form CO_2 and active formate.



(*iii*) Glycine may also get degraded along the succinate-glycine cycle, which gets involved in the synthesis of porphyrins.



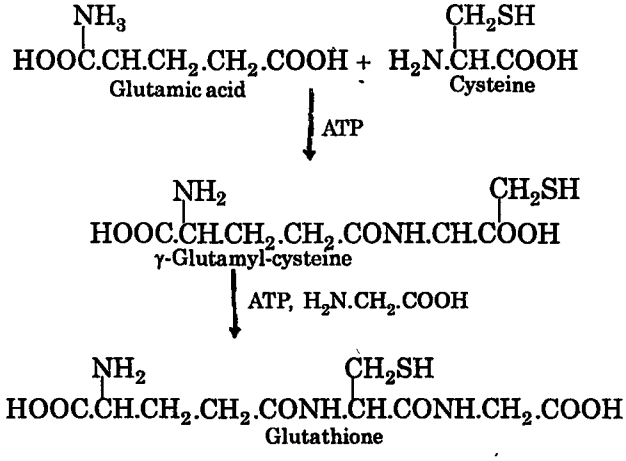
(*iv*) Glycine undergoes condensation with acetyl-CoA to form aminoacetone, which loses amonia to form methylglyoxal. Methyl-glyoxal can be degraded finally to acetyl-CoA.

(*v*) Glycine is also involved in the synthesis of purines.

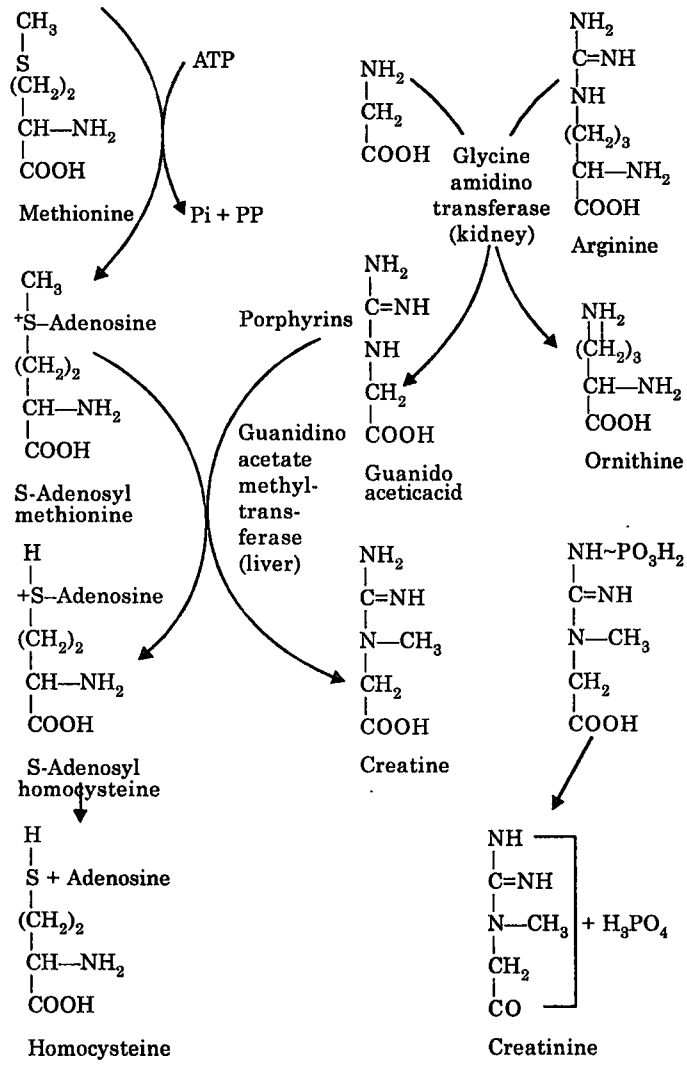
(*vi*) Glycine also combines with potentially harmful substances in the body to form harmless substances which are then excreted in the urine. The process is known as *detoxication* and removes benzoic acid and other aromatic acids (*e.g.*, *p*-amino benzoic acid) absorbed from the alimentary tract or forming during the metabolic processes.

$$C_6H_5.COOH + H_2N.CH_2COOH \longrightarrow \underset{\text{Hippuric acid}}{C_6H_5CO\ NH.CH_2COOH} + H_2O$$

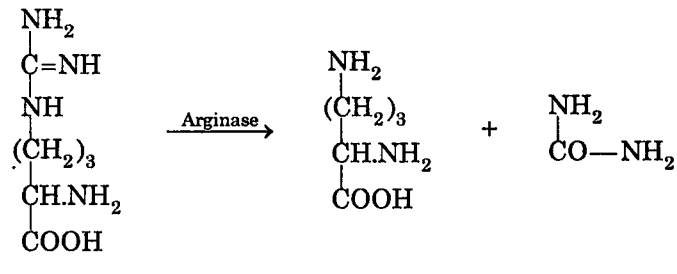
(*vii*) Glycine is also involved in the synthesis of glutathione in which first step involves the combination of glutamic acid and cysteine to form the γ-glutamyl-cysteine which then undergoes condensation with glycine to form glutathion.



(*viii*) Glycine is one of the building blocks in the synthesis of creatine and creatinine. The synthesis involves two steps. In the first step, the amidine group of arginine gets transferred to glycine (transamidination) to form guanidoacetate (glycocyamine) and ornithine. In the second step guanidoacetate gets methylated by activated methionine (S-adenosylmethionine) to form *creatine* (transmethylation).



10. Arginine : Two catabolic roles are known for arginine, *viz.*, formation of creatine (already described in glycine), and formation of urea. Arginine gets degraded to ornithine and urea in the presence of enzyme arginase.



11. Histidine : Histidine is essential for the growth of the young, whereas the adult can synthesise it at an adequate rate. Histidine gets degraded *via* several routes.

(*i*) Histidine on deamination yields urocanate which gets converted to 4-imidazolone-5-propionate. The reaction is catalysed by *urocanase.* The imidazole ring of the 4-imidazolone-5-propionate undergoes hydrolytically cleavage to N-formimino glutamatewhich then transfers its formimino group on the α-carbon atom of tetra-hydrofolate and itself gets converted into glutamate.

(*ii*) In other catabolic pathway, histidine gets decarboxylated in the presence of a decarboxylase to form histamine. It may excrete in the urine as such, as its methyl or acetyl derivative, or may get oxidised by diamine oxidase and aldehyde dehydrogenase to imidazoleacetate, which in turn gets excreted in the urine as such, as a ribotide, or methylated derivative.

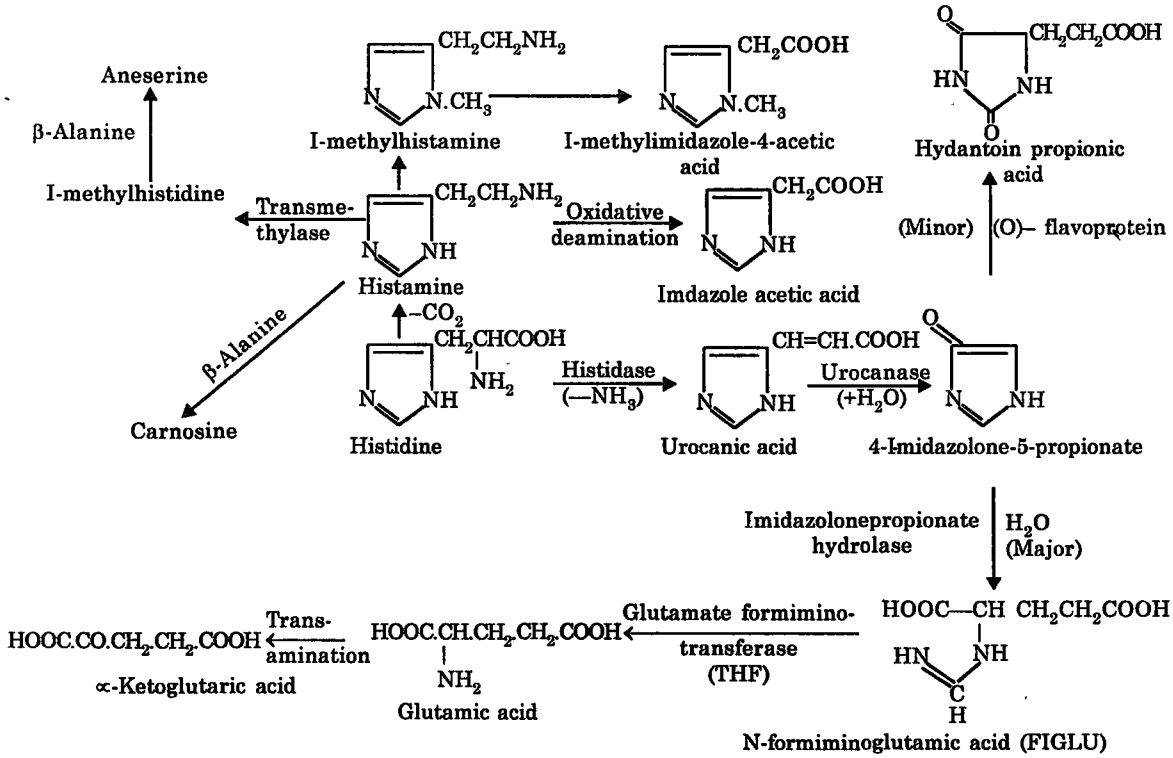
(*iii*) Histidine and its 1-methyl derivative react with β-alanine (from aspartate) to form the pseudodipeptides carnosine and anserine (1-methyl carnosine), respectively.

12. Glutamic acid : Glutamic acid is non-essential amino acid which is readily synthesised from (and converted to) carbohydrate *via* α-ketoglutaric acid.

Glutamic acid gets involved in the synthesis of certain special products, *viz.,* glutathione and glutamine.

The various catabolic routes of glutamic acid involve the conversion to (*i*) *glutamine,* (*ii*) succinate *via* α-ketoglutarate or γ-aminobutyrate, (*iii*) *glutathione,* and (*iv*) *ornithine.*

Glutamic acid is converted to glutamine in the presence of ammonia. This reaction is catalysed by the enzyme glutamine synthetase in presence of ATP and Mg^{2+} ions. The reverse reaction takes place in kidney and is catalysed by the enzyme glutaminase.

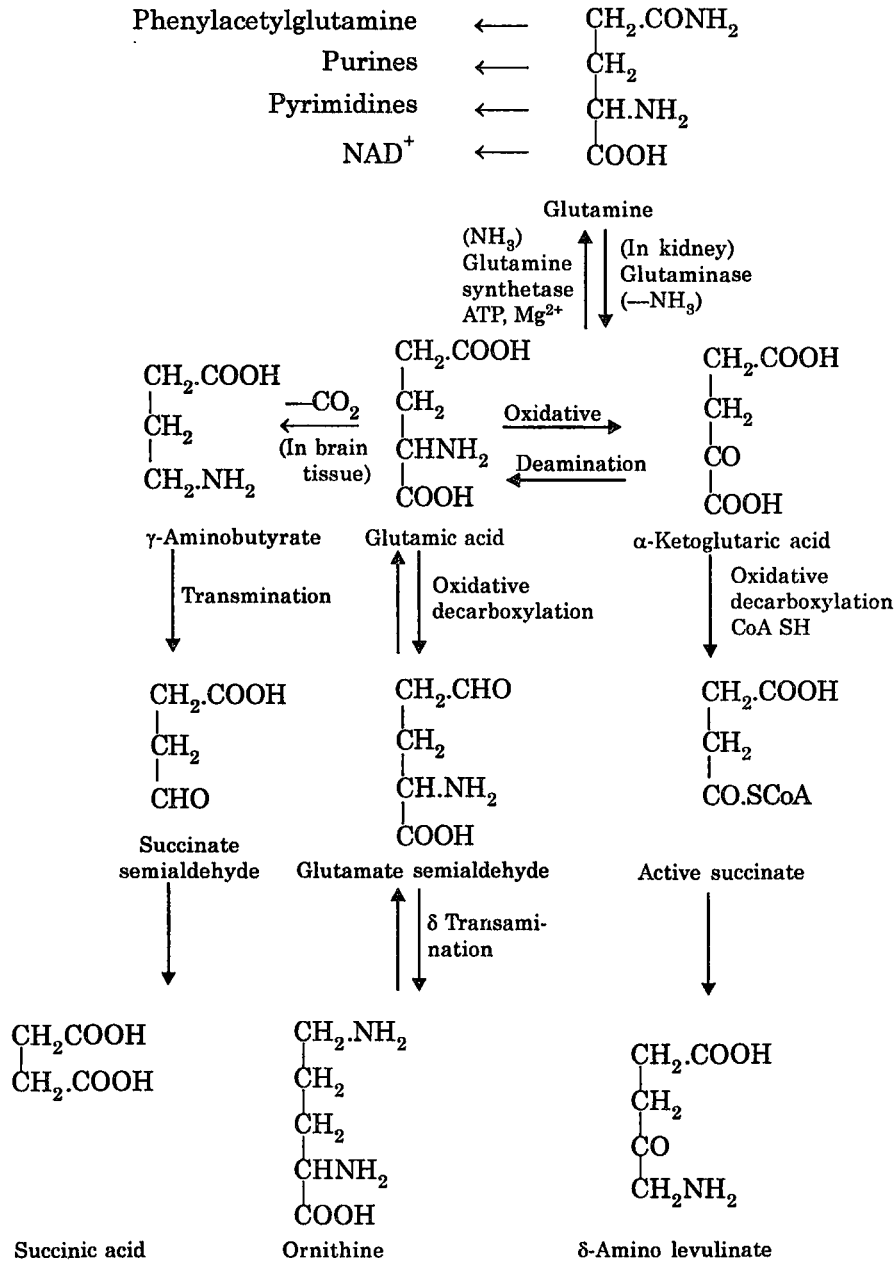


Glutamate is used as a substrate by brain tissue. It first gets decarboxylated to γ-aminobutyrate which then gets transaminated to succinate semialdehyde. The latter on oxidation gets converted to succinate which enters the Kreb's cycle.

On oxidative deamination, glutamate gets converted to α-ketoglutarate which may get oxidative decarboxylated to active succinate in the presence of coenzyme A. It is possible that activated

succinate may enter the Kreb's cycle or it may undergo condensation with glycine to form a β-keto acid which gets decarboxylated spontaneously to δ-aminolevulinate.

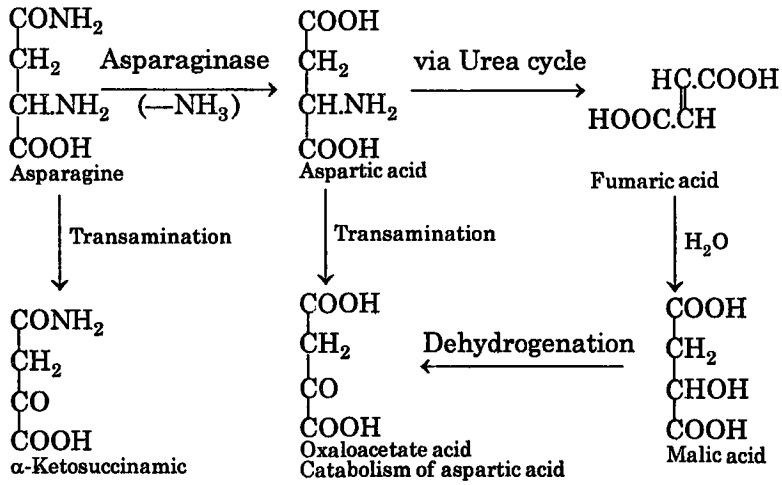
On oxidative decarboxylation, glutamate gets converted to glutamate semialdehyde which on δ-transmination yields ornithine.



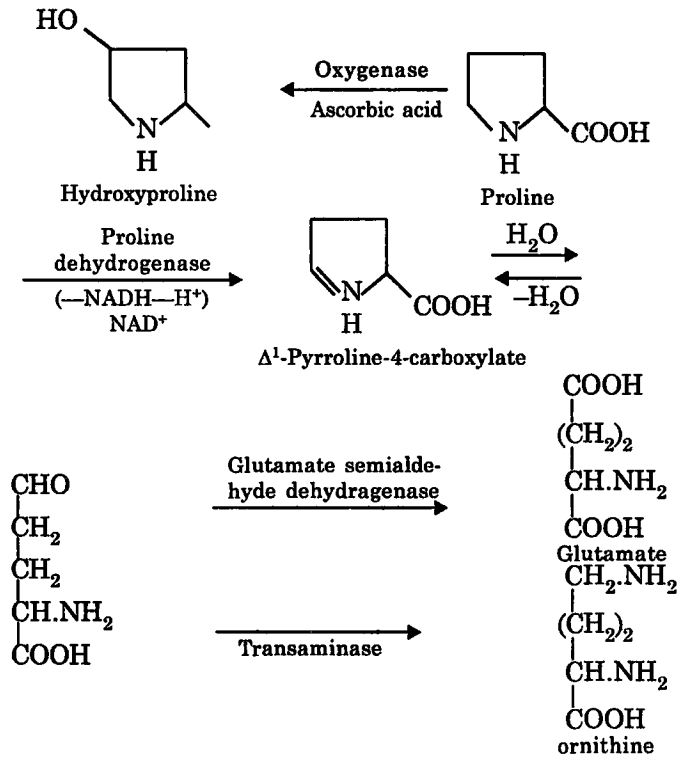
13. Aspartic acid and asparagine : These are the non-essential glucogenic amino acids which are involved in transamination reactions, the ornithine cycle, and in the synthesis of purines and pyrimidines.

Asparagine gets converted into aspartate by asparaginase. Asparate can transfer its amino group to keto acids (transamination) and itself gets converted into oxaloacetate which enters the

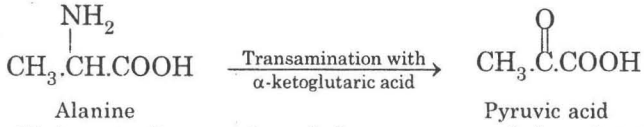
Kreb's cycle. Secondly as in ornithine urea cycle, the asparate molecule on combination with citruline forms argininosuccinate which gets cleaved to arginine and fumarate. The former compound on hydrolysis gives urea, whereas the latter compound gets away from the urea cycle. By the addition of one molecule of water, it (fumarate) is converted into malate which gets dehydrogenated to oxaloacetate (glucogenic compound) :



14. Proline : The main catabolite of proline is glutamate or ornithine in the following way :

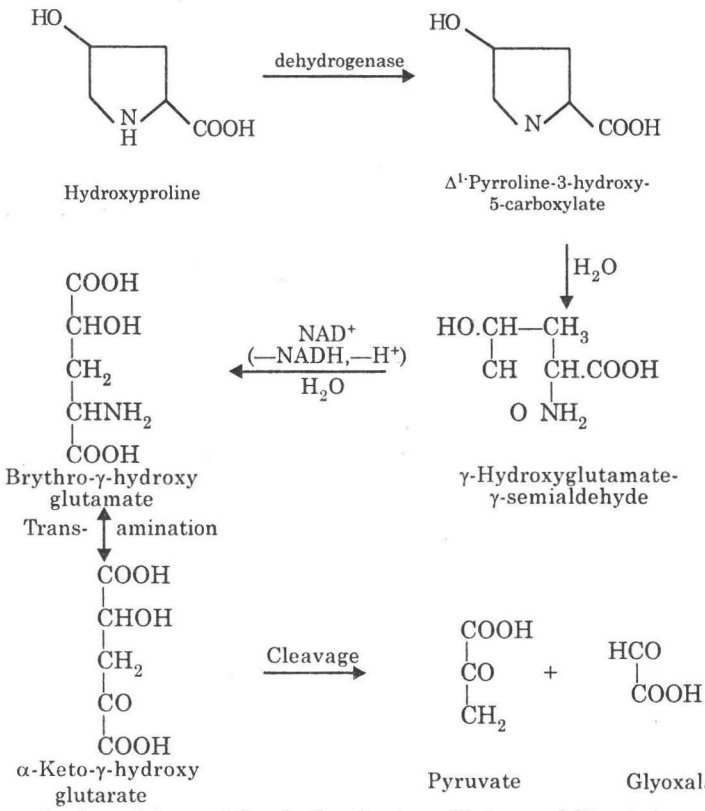


15. Alanine : Alanine gets degraded by involving the usual transamination reaction to pyruvic acid.



Alanine — Pyruvic acid

16. Hydroxyproline: Hydroxyproline gets degraded to pyruvate and glyoxalate in the following manner.



Hydroxyproline → Δ^1-Pyrroline-3-hydroxy-5-carboxylate

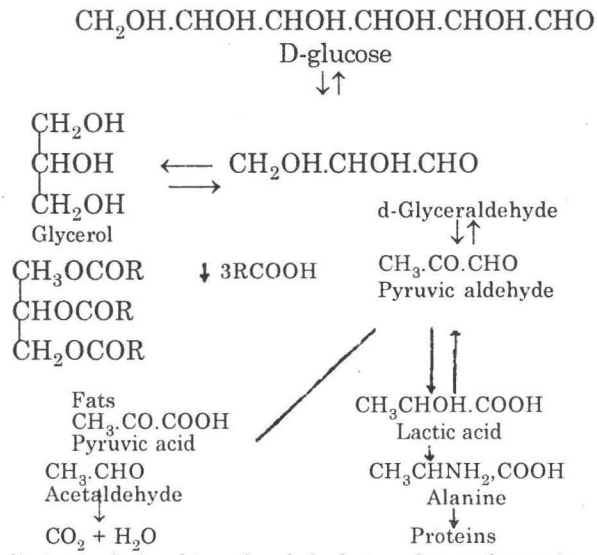
Δ^1-Pyrroline-3-hydroxy-5-carboxylate → γ-Hydroxyglutamate-γ-semialdehyde

γ-Hydroxyglutamate-γ-semialdehyde → Brythro-γ-hydroxy glutamate

Brythro-γ-hydroxy glutamate ⇄ α-Keto-γ-hydroxy glutarate (Trans-amination)

α-Keto-γ-hydroxy glutarate → Pyruvate + Glyoxalate (Cleavage)

Metabolic Interrelationships of Carbohydrates, Fats and Proteins : The biochemical interrelationships of carbohydrates, fats, and proteins are depicted as follows :



Metabolic interrelationships of carbohydrates, fats and proteins

20 Biosynthesis of Urea and Catabolism of Amino Acid Nitrogen

20.1 Introduction

In mammalian tissues, the α-amino groups of amino acids, derived either from the diet or from breakdown of tissue proteins, ultimately get excreted in the urine as urea. It is possible to divide the biosynthesis of urea into 4 processes : (1) transamination, (2) oxidative deamination, (3) ammonia transport, and (4) reactions of the urea cycle. Figure 20.1 relates these areas to overall catabolism of amino acid nitrogen.

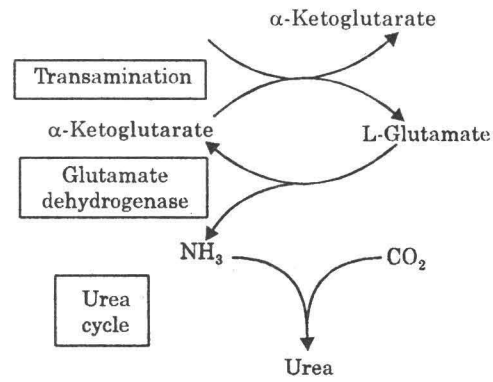


Fig. 20.1 Overall flow of nitrogen in amino acid catabolism. Although the reactions shown are reversible, they are represented as being unidirectional to emphasize the direction of metabolic flow in mammalian amino acid catabolism.

1. Transamination

Transamination is catalyzed by enzymes which are termed *transminases* or *aminotransferases*. This interconverts a pair of amino acids and a pair of keto acids. These generally are α-amino and α-keto acids (Fig. 20.2).

Pyridoxal phosphate forms an essential part of the active site of transaminases and of many other enzymes with amino acid substrates. In all pyridoxal phosphate-dependent reactions of amino acids, the initial step involves formation of an enzyme-bournd Schiff base intermediate.

This intermediate, stabilized by interaction with a cationic region of the active site, can get rearranged in ways that include release of a keto acid with formation of enzyme-bound pyridoxamine phosphate. The bound, amino form of the coenzyme can then form an analogous Schiff base intermediate with a keto acid. During transamination, bound coenzyme thus serves as a carrier of amino groups (Fig. 20.3).

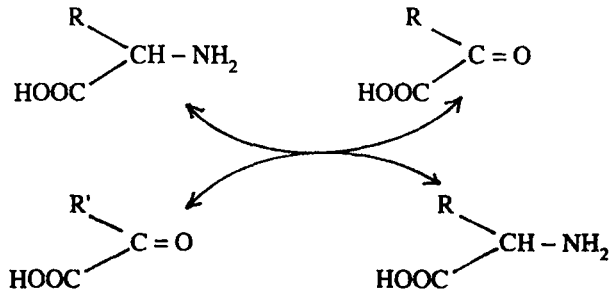


Fig. 20.2. Transmination. The reaction is shown for 2 α-amino and 2 α-keto acids. Non-α-amino or carbonyl groups also participate in transamination, although this is relatively uncommon. The reaction is freely reversible with an equilibrium constant of about 1.

Two transaminases, alanine-pyruvate transaminase (*alanine transaminase*) and glutamate-α-ketoglutarate transaminase (glutamate transaminase), present in most mammalian tissues, catalyze transfer of amino groups from most amino acids to form alanine (from pyruvate) or glutamate (from α-ketogultarate) (Fig. 20.4).

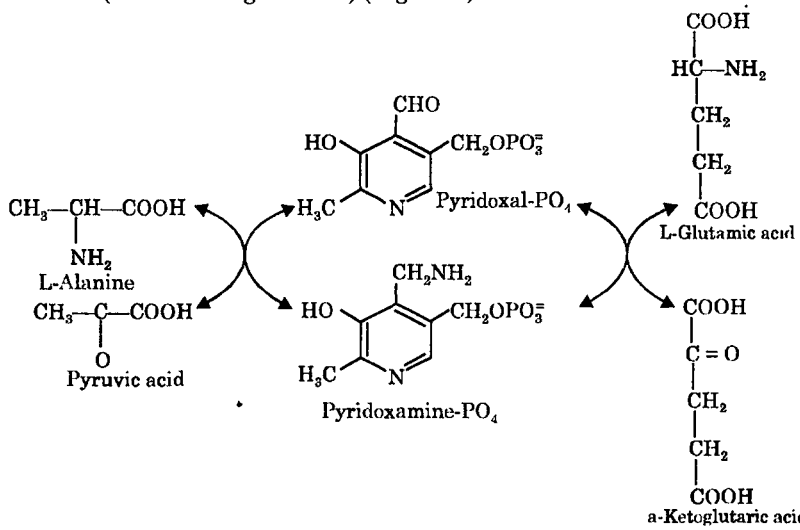
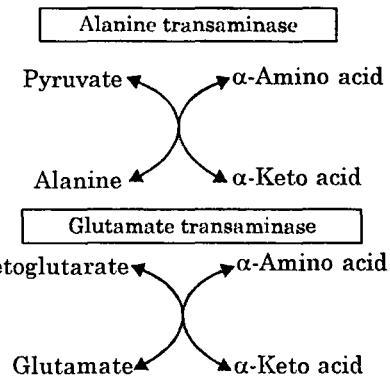


Fig. 20.3. Participation of pyridoxal phosphate in transmination reactions.

As the equilibrium constant for most transaminase reactions is close to unity, transamination is a freely reversible process. This allows transaminases to function both in amino acid catabolism and biosynthesis.



Each transaminase has been found to be specific for the specified pair of amino and keto acids as one pair of substrates but non-specific for the other pair, which may be any of a wide variety of amino acids and their corresponding keto acids. As alanine is also a substrate for glutamate transaminase, all of the amino nitrogen from amino acids that can undergo transamination can be concentrated in glutamate. This is important as L-glutamate is the only amino acid in mammalian *tissues which undergoes oxidative deamination* at an appreciable rate. The

Fig. 20.4. Alanine and glutamate transminases.

formation of ammonia from α-amino groups thus takes place mainly via conversion to the α-amino nitrogen of glutamate.

Most (but not all) amino acids act as substrates for transamination. Exceptions include lysine, threonine, and the cyclic imino acids, proline and hydroxy-proline. Transamination does not get restricted to α-amino groups; the α-amino group of ornithine is readily transaminated, forming glutamate γ-semialdehyde.

2. Oxidative Deamination

Oxidative conversion of many amino acids to their corresponding α-keto acids takes place in homogenates of mammalian liver and kidney tissue. Although most of the activity of homogenates toward L-α-amino acids is ascribed to the coupled action of transaminases plus glutamate dehydrogenase, both L- and D-amino acid oxidase activities do occur in mammalian liver and kidney tissue and are widely distributed in other animals and microorganisms.

Amino acid oxidases are auto-oxidizable flavoproteins, *i.e.*, the reduced FMN or FAD gets reoxidized directly by molecular oxygen forming hydrogen peroxide (H_2O_2) without participation of cytochromes or other electron carriers (Fig. 20.5). The toxic product H_2O_2 is then split to O_2 and H_2O by *catalase,* which takes place widely in tissues, especially liver. Although the amino acid oxidase reactions are reversible, if catalase, is absent the α-keto acid product is nonenzymically decarboxylated by H_2O_2 thereby yielding a carboxylic acid with one less carbon atom.

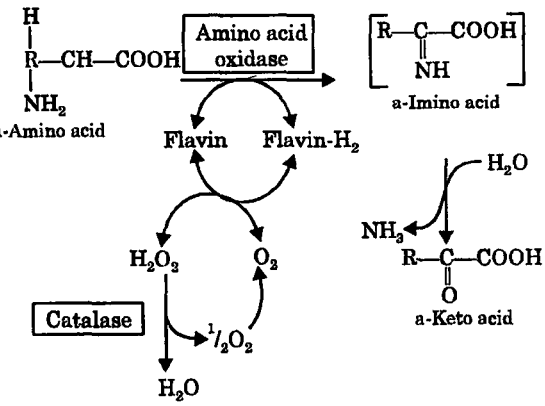


Fig. 20.5. Oxidative deamination catalyzed by L-amino acid oxidase (L-α-amino acid : O_2 oxidoreductase). The α-imino acid, shown in brackets, is not a stable intermediate.

In the amino acid oxidase reactions (Fig. 20.5) the amino acid first gets dehydrogenated by the flavoprotein of the oxidase, forming an α-imino acid. This spontaneously adds water, then decomposes to the corresponding α-keto acid with loss of the α-imino nitrogen as ammonia.

Mammalian L-amino acid oxidase, an FMN-flavoprotein, is restricted to kidney and liver tissue. Its activity is quite low, and it is essentially without effect on glycine or the L-isomers of the dicarboxylic or β-hydroxy-α-amino acids. It thus is not likely that this enzyme fulfills a major role in mammalian anino acid catabolism.

Mammalian D-amino acid oxidase, an FAD-flavoprotein of broad substrate specificity, takes place in the liver and kidney tissue of most mammals. D-Asparagine and D-glutamine are not oxidized, and glycine and the isomers of the acidic and basic amino acids are poor substrates. The physiologic significance of this enzyme in mammals is not known with certainty.

L-glutamate Dehydrogenase

The amino groups of most amino acids ultimately are transferred to α-ketoglutarate by transamination, forming glutamate (Fig. 20.1). Release of this nitrogen as ammonia is catalyzed by L-glutamate dehydrogenase, an enzyme of high activity widely distributed in mammalian tissues (Fig. 20.6). Liver glutamate dehydrogenase is a regulated enzyme whose activity gets affected by allosteric modifiers such as ATP, GTP, and NADH, which inhibit the enzyme; and

ADP, which activates the enzyme. Certain hormones appear also to influence glutamate dehydrogenase activity.

Glutamate dehydrogenase uses either NAD^+ or $NADP^+$ as cosubstrate. The reaction is reversible and functions both in amino acid catabolism and biodynthesis. It therefore functions not only to funnel nitrogen from glutamate to urea (catabolism) but also to catalyze amination of α-ketoglutarate by free ammonia.

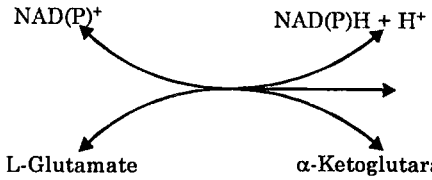


Fig. 20.6. The L-glutamate dehydrogenase reaction. The designation $NAD(P)^+$ means that either NAD^+ or $NADP^+$ can serve as cosubstrate. The reaction is reversible but the equilibrium constant favours glutamate formation.

Formation of Ammonia

In addition to ammonia formed in the tissues, a considerable quantity by intestinal bacteria is formed from dietary protein and from urea present in fluids secreted into the gastrointestinal tract. This ammonia gets absorbed from the intestine into the portal venous blood, which characteristically contains higher levels of ammonia than does systemic blood.

Under normal circumstances the liver promptly removes the ammonia from the portal blood, so that blood leaving the liver (and indeed all of the peripheral blood) is virtually ammonia-free. This is essential because even minute quantities of ammonia are toxic to the central nervous system. The symptoms of ammonia intoxication include a peculiar flapping tremor, slurring of speech, blurring of vision, and in severe cases, coma and death.

The ammonia content of the blood in renal veins exceeds that in renal arteries, revealing that the kidneys produce ammonia and add it to the blood. However, the excretion into the urine of the ammonia produced by renal tubular cells constitutes a far more significant aspect of renal ammonia metabolism. Ammonia production, an important renal tubular mechanism for regulation of acid-base balance and conservation of cations, gets markedly increased in metabolic acidosis and depressed in alkalosis. This ammonia is derived, not from urea, but from intracellular amino acids, particularly glutamine. Ammonia release is catalyzed by renal *glutaminase* (Fig. 20.7).

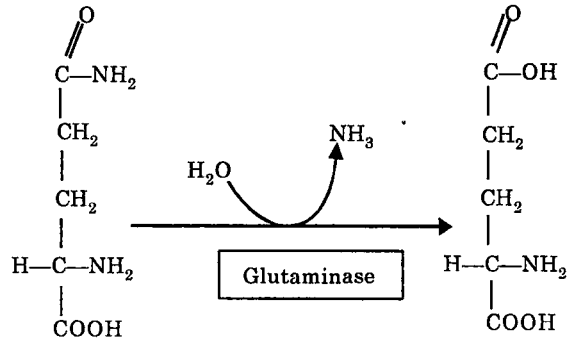


Fig. 20.7. The glutaminase reaction proceeds essentially irreversibly in the direction of glutamate and NH_3 formation.

3. Transport of Ammonia

Although ammonia may be excreted as ammonium salts—especially in metabolic acidosis—the vast majority gets excreted as urea, the principal nitrogenous component of urine. Ammonia constantly produced in the tissues but present only in trace in peripheral blood (10-20 μg/dL),gets rapidly removed from the circulation by the liver and converted to

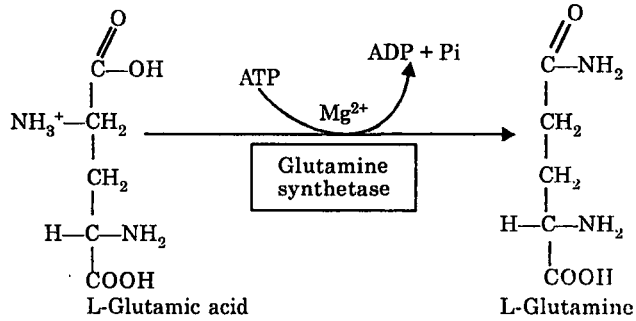


Fig. 20.8. The glutamine synthetase reaction. This reaction strongly favours glutamine synthesis.

glutamate, to glutamine, or to urea. These trace levels of ammonia in blood contrast sharply with the more considerable quantities of free amino acids, especially glutamine.

Removal of ammonia via glutamate dehydrogenase was mentioned above. Formation of glutamine gets catalyzed by glutamine synthetase (Fig. 20.8), a mitochondrial enzyme which is present in highest quantities in renal tissue. Synthesis of the amide bond of glutamine gets accomplished at the expense of hydrolysis of one equivalent of ATP to ADP and P_i. The reaction is thus strongly favoured in the direction of glutamine synthesis.

Liberation of the amide nitrogen of glutamine as ammonia takes place, not by reversal of the glutamine synthetase reaction, but by hydrolytic removal of ammonia catalyzed by *glutaminase* (Fig. 20.8). The glutaminase reaction, unlike the glutamine synthetase reaction, does not involve adenine nucleotides, strongly favours glutamate formation, and does not function in glutamine synthesis.

Glutamine synthetase and glutaminase (Fig. 20.9) thus catalyze interconversion of free ammonium ion and glutamine in a manner reminiscent of the interconversion of glucose and glucose 6-phosphate by glucokinase and glucose-6-phosphatase. An analogous reaction gets catalyzed by asparaginase of animal, plant and microbial tissue. Asparaginase and L-glutaminase have both been employed as antitumor agents because certain tumors exhibit abnormally high requirements for glutamine and asparagine.

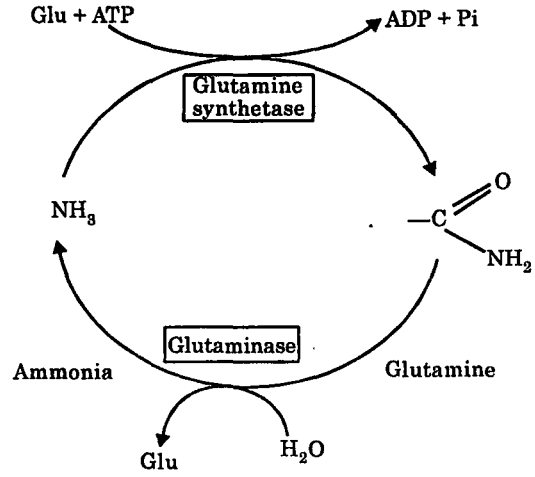


Fig. 20.9. Interconversion of ammonia and of glutamine catalyzed by glutamine synthetase and glutaminase. Both reactions are strongly favoured in the directions indicated by the arrows. Glutaminase thus serves solely for glutamine deamidation and glutamine synthetase solely for synthesis of glutamate (Glu = glutamate).

Whereas in brain the major mechanism for removal of ammonia is glutamine formation, in the liver the most important pathway is urea formation. Brain tissue can form urea, although this does not play a significant role in ammonia removal. Formation of glutamine in the brain must be preceded by synthesis of glutamate in the brain because the supply of blood glutamate is inadequate in the presence of high levels of blood ammonia. The immediate precursor is α-ketoglutarate. This would rapidly deplete citric acid cycle intermediates unless they could be replaced by CO_2 fixation with conversion of pyruvate to oxaloacetate.

A significant fixation of CO_2 into amino acids does indeed take place in the brain, presumably by way of the citric acid cycle, and after infusion of ammonia more oxaloacetate is diverted to the synthesis of glutamine (rather than to aspartate) via α-ketoglutarate.

4. Biosynthesis of Urea

A moderately active man consuming about 300 g of carbohydrate, 100 g of fat, and 100 g of protein daily must excrete abut 16.5 g of nitrogen daily. Ninety-five percent get eliminated by the kidneys and the remaining 5% in the stool. The major pathway of nitrogen excretion in humans is as urea which is synthesized in the liver, released into the blood, and cleared by the kidneys. In humans eating an accidental diet, urea forms 80-90% of the nitrogen excreted.

Reactions of the Urea Cycle

The reactions and intermediates in biosynthesis of 1 mol of urea from 1 mol each of ammonia, carbon dioxide (activated with Mg^{2+} and ATP), and of the α-amino nitrogen of aspartate have been depicted in Fig. 20.10. The overall process needs 3 mols of ATP (2 of which are converted to ADP + Pi and 1 to AMP + PPi, and the successive participation of 5 enzymes catalyzing the numbered reaction of Fig. 20.10. Of the 6 amino acids involved in urea synthesis, one (N-acetylglutamate) functions as an enzyme activator rather than as an intermediate. The remaining 5—aspartate, arginine, ornithine, citrulline, and argininosuccinate—all function as carriers of atoms which ultimately become urea. Two (aspartate and arginine) are found in proteins, the remaining 3 (ornithine, citrulline and argininosuccinate) do not. The major metabolic role of these latter 3 amino acids in mammals is urea synthesis. Note that urea formation is in part a *cyclical process.* The ornithine used in reaction 2 is regenerated in reaction 5. There occurs thus no net loss or gain of ornithine, citrulline, argininosuccinate, or arginine during urea synthesis; however, ammonia, CO_2, ATP, and aspartate are consumed.

Reaction 1 : Synthesis of carbamoyl phosphate : Condensation of 1 mol each of ammonia, carbon dioxide, and phosphate (derived from ATP) to form carbamoyl phosphate gets catalyzed by *carbamoyl phosphate synthetase,* an enzyme present in liver *mitochondria* of all ureotelic organisms, including humans. The 2 mol of ATP hydrolyzed during this reaction provide the driving force for synthesis of 2 covalent bonds—the amide bond and the mixed carboxylic acid-phosphoric acid anhydride bond of carbamoyl phosphate. In addition to Mg^{2+}, a dicarboxylic acid, preferably N-acetylglutamate, is required. Although the exact role of N-acetylglutamate, is not known with certainty, yet its presence causes a profound conformational change in the structure of carbamoyl phosphate synthetase which exposes certain sulfhydryl groups, conceals others, and affects the affinity of the enzyme for ATP.

In bacteria, glutamine rather than ammonia acts as a substrate for carbamoyl phosphate synthesis. A similar reaction catalyzed by carbamate kinase is also important in citrulline utilization by bacteria.

Reaction 2 : Synthesis of citrulline : Transfer of a carbamoyl moiety from carbamoyl phosphate to ornithine, forming citrulline + Pi gets catalyzed by *ornithine transcarbamoylase* of liver mitochondria. The reaction is highly specific for ornithine, and the equilibrium strongly favours citrulline synthesis.

Reaction 3 : Synthesis of argininosuccinate : In the argininosuccinate synthetase reaction, aspartate and citrulline get linked together via the amino group of aspartate. The reaction needs ATP, and the equilibrium strongly favours argininosuccinate.

Reaction 4 : Cleavage of argininosuccinate to arginine and fumarate : Reversible cleavage of argininosuccinate to arginine plus fumarate gets catalyzed by *argininosuccinase,* a cold-labile enzyme of mammalian liver and kidney tissues. Loss of activity in the cold, associated with dissociation into 2 protein components, is prevented by Pi, arginine and argininosuccinate or *p*-hyroxymercuribenzoate, which has no adverse effect on activity. The reactive occurs via a *trans* elimination mechanism. The fumarate formed may be converted to oxaloacetate via the fumarase and malate dehydrogenase reactions and then transaminated to regenerate aspartate.

Reaction 5 : Cleavage of arginine to ornithine and urea : This reaction completes the urea cycle and regenerates ornithine, a substrate for reaction 2. Hydrolytic cleavage of the guanidine group of arginine gets catalyzed by *arginase,* found in the livers of all ureotelic organisms. Smaller quantities of arginase are also found in renal tissue, brain, mammary gland, testicular tissue,

and skin. Mammalian liver arginase is activated by Co^{2+} or Mn^{2+}. Ornithine and lysine are potent inhibitors competitive with arginine.

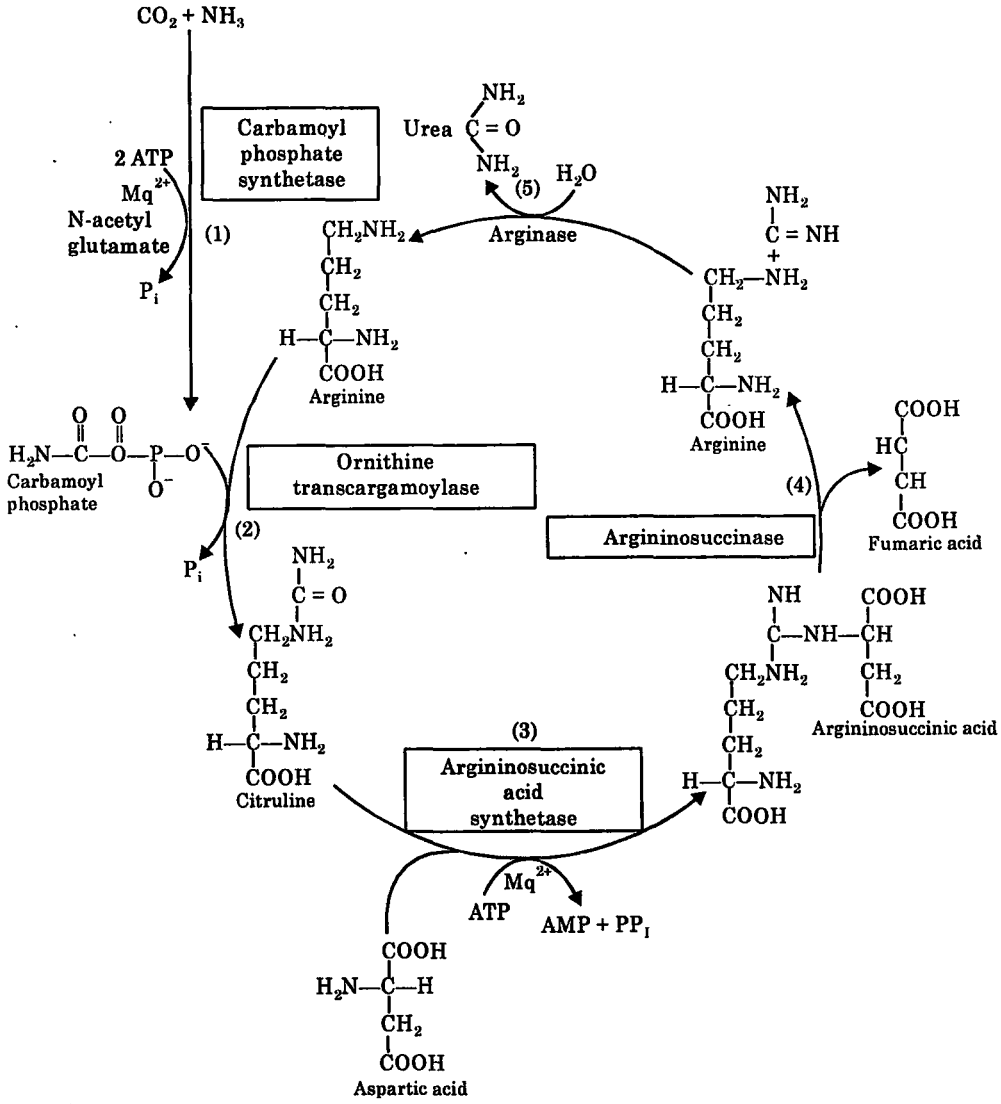


Fig. 20.10. Reactions and intermediates of urea biosynthesis.

20.2 Regulation of Urea Synthesis

Carbamoyl phosphate synthetase acts with mitochondrial glutamate dehydrogenase to channel nitrogen from glutamate (and hence from all amino acids; see Fig. 20.10) into carbamoyl phosphate and thus into urea. While the equilibrium constant of the glutamate dehydrogenase reaction favours glutamate rather than ammonia formation, removal of ammonia by carbamoyl phosphate synthetase and oxidation of α-ketoglutarate serve to favour glutamate catabolism. This effect is enhanced by ATP, which, in addition to being a substrate for carbamoyl phosphate synthesis, stimulates glutamate dehydrogenase activity, unidirectionally favouring ammonia formation.

21 Metabolism of Lipids

21.1 Introduction

The *lipids* or *fats* are a heterogenous group of substances, but are essentially hydrocarbons in nature. Catabolism of hydrocarbons yields more energy than the catabolism of most other organic compounds. From an energy viewpoint, the component fatty acids are the most important part.

Recent studies have revealed major differences in the lipid components and fatty acyl chains of prokaryotic and eukaryotic organisms. The fatty acids of bacteria are generally 10-20 carbons in length, with 15-19 carbon chains predominating. These are mainly of four types : (1) straight chain saturated, (2) straight chain mono-unsaturated, (3) branched chains and (4) cyclopropane fatty acids.

Most of the longer chain polyunsaturated fatty acids especially C_{18} di- and tri-unsaturated acids, which are commonly found in the lipids of animal tissues, are absent in bacteria. The predominant mono unsaturated fatty acids are *palmitoleic acid,* 16 : $1\Delta^9$ and *cis-vaccenic acid,* 18 : $1\Delta^{11}$, but not *oleic acid,* 18 : $1\Delta^9$. Branched chain fatty acids have also been found in a number of bacterial species. Some examples include *10-methyloctadecanoic* and *in Mycobacterium tuberculosis* and *12-methyltradeconic* and *isopalmitic acid* in the Bacillaceace and Micrococcaceae.

Cyclopropane fatty acids are widely distributed among both gram positive and gram negative bacteria. The predominant cyclopropane fatty acids are *cis-9, 10-methylenehexadecanoic acids* are *cis-9, 10-methylenehexadecanoic acid* and *cis-11, 12-methyleneoctadecanoic acid* (lactobacillic acid), *Cis-9, 10-methylenehexadecanoic acid* (dihydrosterculic acid) has also been found.

21.2 Fat Oxidation

In the natural system, the enzyme lipase brings about the bydrolysis of fats into glycerol and fatty acids before oxidation.

$$\text{Fats} + H_2O \xrightarrow{\text{lipase}} \text{glycerol} + \text{fatty acids}$$

Glycerol on reacting with ATP forms glycerol phosphate which gets oxidized to glyceraldehyde-3-phosphate. Glycogen or glucose and fructose may be synthesized from this by the reversal of glycolytic process. Alternatively, it could be converted to pyruvate and then oxidized by TCA cycle.

Oxidation of fatty acids is more or less a sequential process for which the following two pathways have been postulated :

1. β-Oxidation of fatty acids : It was discovered by F. Knoop in the beginning of twentieth century. Initially this process was discovered and explained in animal systems. Later on, the enzymes involved in the process were also discovered in plants. It involves the following steps :

(i) The initial step in β-oxidation is the activation of a fatty acid by its transformation into the corresponding CoA-thioester with the help of coenzyme A. The energy required for this process is derived from ATP. This reaction has been found to be catalysed by the appropriate *acyl-CoA synthetase* (an enzyme).

$$RCH_2CH_2COOH + CoASH \xrightarrow{\text{ATP AMP + PPi}} RCH_2CH_2\overset{\overset{O}{\|}}{C}.SCoA$$

Many such enzymes are known which have been named according to the length of the carbon chain of the fatty acid that reacts most rapidly. Generally, two types of such enzyems are known, one specific for medium length carbon chains (4C to 12C) and the other for longer chains. For example, the enzyme from *Bacillus megatherium* reacts with fatty acids of 6 to 20 carbon atoms.

Another mechanism for the synthesis of an acyl —CoA derivatives involves the transfer reaction catalysed by *thiophorases.* It is transacetylation reaction wherein CoA gets transferred from propionyl-CoA or succinyl-CoA to the appropriate fatty acid :

$$\text{Propionyl—CoA} + RCOOH \rightleftharpoons \text{Propionic acid} + R\text{—COSCoA}$$

(ii) The product formed in the initial step (*i*) undergoes dehydrogenation, *i.e.,* two hydrogen atoms are removed from α and β carbon atoms of the fatty acids to form α, β-unsaturated fatty acyl-CoA derivatives. They require the coenzyme FAD and enzyme, acyl-CoA dehydrogenase.

$$RCH_2CH_2\overset{\overset{O}{\|}}{C}.SCoA \xrightarrow[FAD\ FADH_2]{\text{dehydrogenase}} R\overset{H}{\underset{|}{C}}{=}\overset{H}{\underset{|}{C}}.\overset{\overset{O}{\|}}{C}.SCoA$$

Three types of dehydrogenases have been identified from liver tissues. They have been found to differ according to their specificity to the substrate. The first has been found to be acting on long chain fatty acids (C_{14}, C_{16}, C_{18}), whereas the other two have been found to be acting on medium or short chains.

(iii) The product formed in the step (*ii*) involves addition of water in the presence of the enzyme enoyl-CoA hydrase and yields a β-hydroxyacyl-CoA derivative.

$$R\overset{H}{\underset{|}{C}}{=}\overset{H}{\underset{|}{C}}.\overset{\overset{O}{\|}}{C}SCoA + H_2O \xrightleftharpoons{\text{hydrase}} R\overset{H}{\underset{|}{C}}\overset{OH}{\underset{|}{H}}CH_2\overset{\overset{O}{\|}}{C}.SCoA$$

This hydration is very often stereospecific as the *trans* isomer is converted to the L (+) isomer and the *cis* isomer to the D (–)- isomer.

(iv) The β-hydroxy group of the product formed in *(iii)* gets dehydrogenated to the keto group by the enzyme, β-hydroxy—CoA dehydrogenase thereby yielding the corresponding β-ketoacetyl CoA ester. The hydrogen is accepted by NAD^+. A single enzyme attacks fatty acids of different lengths.

$$RCH(OH).CH_2.C(=O).SCoA + NAD^+ \xrightleftharpoons{\text{dehydrogenase}} RC(=O).CH_2C(=O).SCoA + NADH + H^+$$

This NADH get reoxidised through the electron transport chain and forms 3 molecules of ATP.

(v) The final step involves the oxidation of thiolytic cleavage of the ketoacyl—CoA by β-ketothiolase, thereby forming acetyl—CoA.

$$RC(=O).CH_2C(=O).SCoA + HSCoA \longrightarrow RC(=O)SCoA + CH_3C(=O).SCoA$$

In this reaction the product is a fatty acid with two carbon atoms short. It may enter the entire process of oxidation at step *(ii)* and further dissociation may take place. Hence, it is a process of cleaving fatty acids into fragments of 2 C compounds in steps (Fig. 21.1).

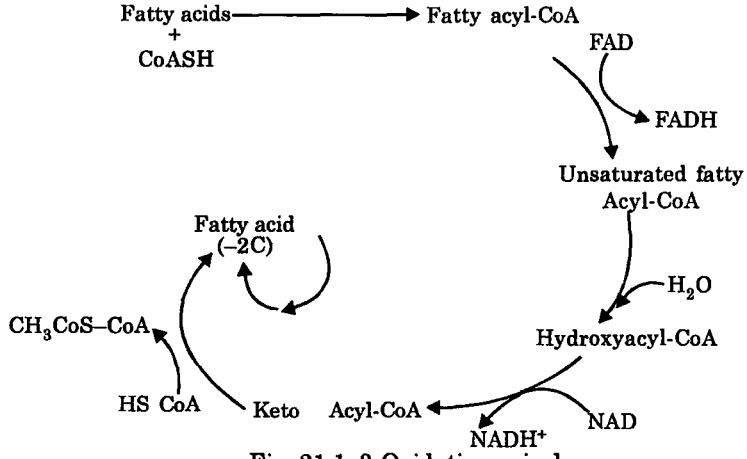


Fig. 21.1. β-Oxidation spiral.

In summary the shortening of fatty acyl—CoA two carbon atoms can be represented by the following equation :

$$RCH_2CH_2COSCoA + FAD + NAD^+ + HSCoA \longrightarrow RCOSCoA + CH_3COSCoA + FADH_2 + NADH + H^+$$

The β-oxidation of palmitic acid is illustrated in Fig. 21.2.

β-Oxidation of fats is a good source of energy production. In each cycle of β-oxidation, there occurs the formation of 5 ATP molecules (3 from the oxidation of NADH and 2 from $FADH_2$). One ATP gets used up in the reaction. In this way 35 – 1 = 34 ATP molecules are formed in 7 turns of palmitic acid oxidation. Besides this, 8 acyl CoA produced from palmitic acid will yield 12 × 8 = 96 ATP molecules. This will give a total of 130 (96 + 34) ATP per palmitic acid molecule.

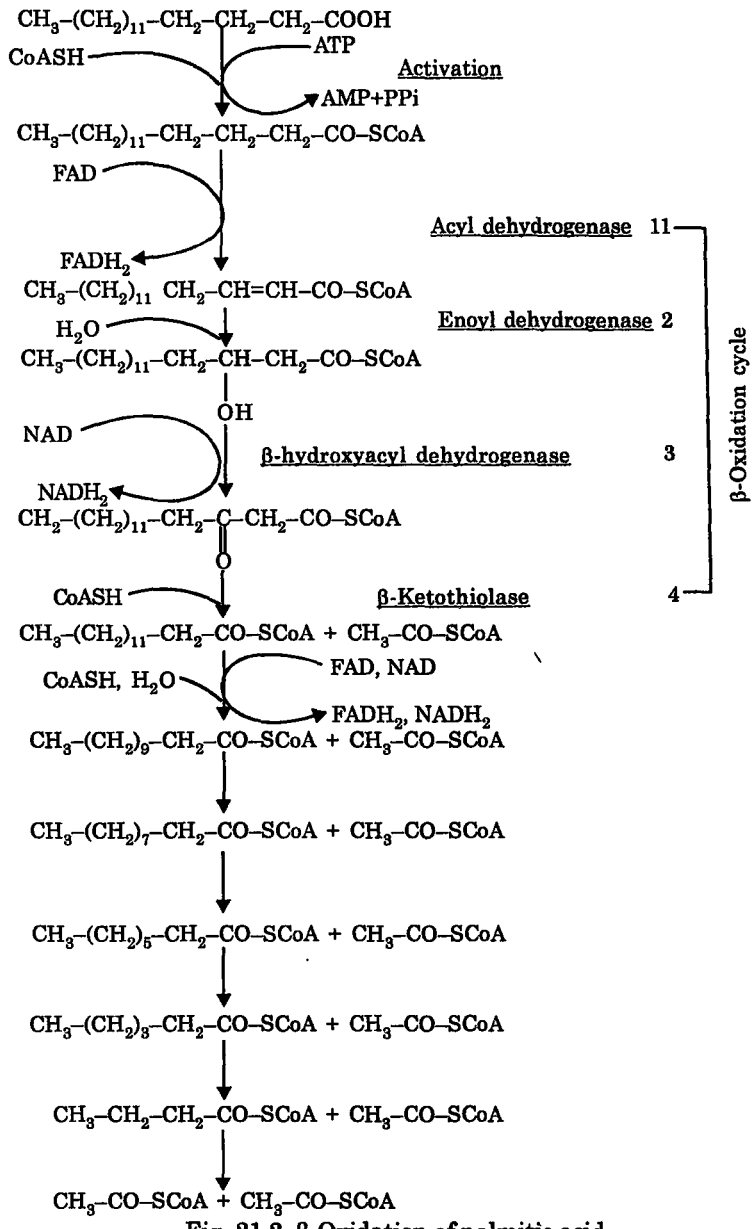


Fig. 21.2. β-Oxidation of palmitic acid.

The end product of β-oxidation of fatty acid is acetyl-CoA. Fatty acids having odd number of carbon atoms form acetyl-CoA and propionyl-CoA. The acetyl-CoA has been either oxidized for the production of ATP or converted into carbohydrates. The propionyl CoA gets converted to malonic semialdehyde in both plants and animals. Malonic semialdehyde could be converted to alanine by transamination with glutamic acid in animals. In plants, however, this compound gets oxidised to CO_2 and H_2O.

2. Alpha-oxidation of fatty acids : P.K. Stumpf postulated an alternative mechanism for fatty acid oxidation. In the first step, the fatty acid first gets decarboxylated and then reduced to an aldehyde having one carbon less than the original fatty acid. This reaction has been catalysed by

aldehyde having one carbon less than the original fatty acid. This reaction has been catalysed by a peroxidase.

$$RCH_2CH_2CH_2COOH \xrightarrow[\text{fatty acid peroxidase}]{+H_2O_2} RCH_2CH_2CHO + CO_2 + H_2O + O_2$$

This aldehyde then gets oxidized to the acid form by the enzyme dehydrogenase.

$$RCH_2CH_2CHO \xrightarrow[\text{aldehyde dehydrogenase}]{NAD\ NADH + H^+} RCH_2CH_2COOH$$

This new acid may act as a substrate for fatty acid peroxidase and may undergo another turn in the α-oxidation spiral. This kind of α-oxidation is seen in the peanut cotyledones. In leaf tissues and brain microsomal preparations, a different type of α-oxidation has been reported, in which decarboxylation occurs after oxidation of the fatty acid.

The biological significance of α-oxidation is not known with certainty. However, it may produce fatty acids with odd number of carbon atoms. Further, the aldehydes formed during this process may get reduced so as to form long chain alcohols.

21.3 Conversion of Fats into Carbohydrates

There are some fat storing seeds, bacteria and fungi in which fats are converted readily into sucrose and other complex sugars. Some of the carbon atoms of the fats are also converted into amino acids. The process called *glyoxylate cycle* is carried out in special cell organelles called *glyoxysomes*. However, it is important to be emphasized that glyoxysomes are present only in those tissues which convert fat into carbohydrate. It was H.L. Kornberg of Oxford university who worked out on this cycle in the late 1950s.

In this cycle, first of all acetyl-CoA derived from the mitochondrial fatty acid oxidation gets diffused to the *glyoxysomes*. In these, acetyl CoA may condense, with oxaloacetate to form citrate involving the enzyme *citrate synthetase*. Then citrate gets isomerised to isocitrate by the enzyme aconitase. Now isocitrate may get converted to malate through Kreb's cycle intermediate, thereby forming CO_2. However, in glyoxysomes, it gets cleaved primarily to succinate and glyoxylate by the enzyme called isocitrase. Out of these, succinate may be involved in the synthesis of sugars and amino acids by interacting with some of the Kreb's cycle intermediates and by the reversal of glycolytic pathway. On the other hand, the other product called glyoxylate may interact with another molecule of acetyl-CoA thereby forming malate, in the presence of enzyme, malate synthetase. At a later stage, malate dehydrogenate brings about oxidation of malate to oxalacetate, which may undergo another round of acylation to form citrate. The glyoxylate has been depicted in Fig. 21.3.

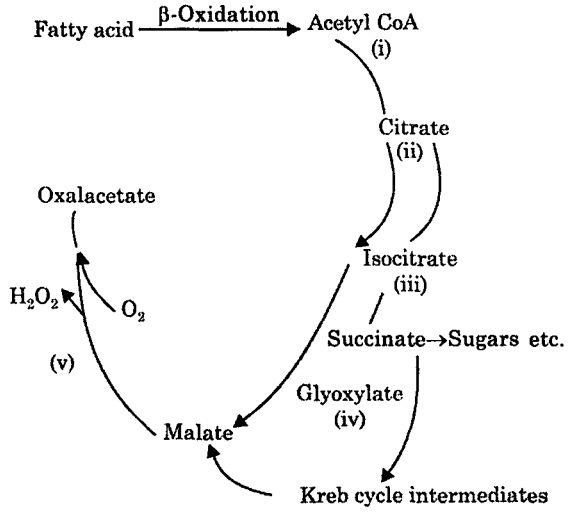


Fig. 21.3. Glyoxylate cycle. Enzymes—(*i*) Citrate synthetase, (*ii*) Aconitase, (*iii*) Isocitratase, (*iv*) Malate synthetase, (*v*) Malate dehydrogenase.

The overall result of glyoxylate cycle involves the conversion of 2 moles of

the synthesis of proteins and sugars. Had no cycle been operated, isocitrate formed in the second step (Fig. 8.3) would have been converted into malate via succinate and fumarate during which carbons are lost as CO_2. In the glyoxylate cycle, these CO_2 yielding reactions are not taking place and the 2 carbons of acetyl—CoA remain conserved.

21.4 Biosynthesis of Fats

Fats are synthesised from fatty acids and glycerol by the reversal of hydrolytic process.

1. Biosynthesis of saturated fatty acids : It was believed for long that fatty biosynthesis was the reversal of the pathway of fatty acid degradation. However, several major points of difference between the degradation and biosynthesis of fatty acids are known. These are given as follows :

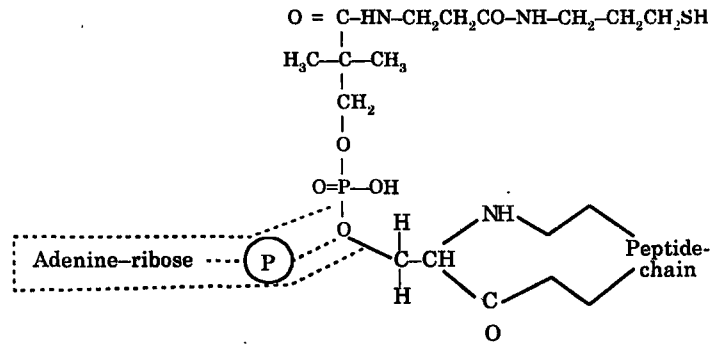


Fig. 21.4. Structure of the prosthetic group of ACP and CoA. In ACP the 4'-phosphopantetheine is linked to the peptide chain via a serine residue.

(i) CoA-derivatives do not act as the substrates of the enzymes in fatty acid synthesis but instead acyl moieties are connected to an *acyl carrier protein* (ACP). ACP has a molecular weight of 10,000. Its prosthetic group is 4'-phosphopantetheine and it thus resembles coenzyme A (Fig. 21.4).

(ii) In biosynthesis, the basic "adding unit" is malonyl-CoA but not acetyl-CoA.

(iii) Biosynthesis uses NADPH while NAD is involved in oxidation. In addition ATP and bicarbonate are also employed.

(iv) In biosynthesis, the hydroxy acids involved are D(–)-β-isomers while the L(+)-β isomers occur in oxidation.

Even numbered saturated fatty acids are synthesized by a series of reactions in which 2 carbon units derived from malonyl-ACP molecule. The initial reactions in fatty acid synthesis involves the formation of acetyl-ACP and malonyl-ACP :

$$\text{(i)}\ CH_3—CO—SCoA + ACP \underset{\text{transacetylase}}{\overset{\text{acetyl}}{\rightleftharpoons}} CH_3—CO—SACP + CoASH$$

$$\text{(ii)}\ CH_3—CO—SCoA + ATP + CO_2 \underset{\text{carboxylase}}{\overset{\text{acetyl-CoA}}{\rightleftharpoons}} \underset{\displaystyle COOH}{\overset{\displaystyle CH_2}{|}}—CO—SCoA + ADP + Pi$$

$$\text{(iii)}\ \underset{\displaystyle COOH}{\overset{\displaystyle CH_2}{|}}—CO—SCoA + ACP \underset{\text{transacetylase}}{\overset{\text{malonyl}}{\rightleftharpoons}} \underset{\displaystyle COOH}{\overset{\displaystyle CH_2}{|}}—CO—SACP + CoASH$$

The formation of malonyl-CoA has been catalyzed by the biotincontaining enzyme called *acetyl-CoA carboxylase.* In some micro-organisms, malonyl-CoA can be formed by reactions other than carboxylation of acetyl CoA. These reactions are as follows :

(i) Activation of malonate by the specific *malonyl-CoA synthetase* in the presence of ATP and CoA,

$$\text{Malonate} + \text{CoASH} + \text{ATP} \longrightarrow \text{malonyl—CoA} + \text{ADP} + \text{Pi}$$

(ii) CoA transferase reaction between malonate and succinyl-CoA or acetoacetyl-CoA,

$$\text{R—CO—SCoA} + \text{malonate} \rightleftharpoons \text{malonyl—CoA} + \text{RCOOH and}$$

(iii) Oxidation of malonyl-semialdehyde to malonyl CoA.

An attempt was made to study fatty acid synthesising systems from a number of resources such as *E.coli*, yeast and pigeon liver. In *E. coli*, there are seven different enzymes which are involved in each turn of a cycle so as to extend the fatty acyl chain. Three of these are *acetyl transacetylase, acetyl CoA carboxylase* and *malonyl transacetylase* which are involved in the formation of acetyl-ACP and malonyl-ACP. Four additional enzymes are needed to elongate acyl-SACP by two carbon atoms to form a 4-carbon acyl fragment, the butyryl-SACP. These steps are as follows :

(i) Firstly, acetyl-ACP reacts with malonyl ACP to form acetoacetyl SACP in the presence of 3-*ketoacyl SACP synthetase.*

(ii) Secondly, acetoacetyl-SACP is reduced to D(–)-β-hydroxy-butyryl-SACP by the enzyme *3-ketoacyl SACP reductase.*

(iii) Dehydration of D(–)-β-hydroxybutyryl—SACP with the enzyme β-*hedroxyacy—ACP dehydrogenase* yields crotonyl-SACP.

(iv) Reduction of crotonyl-SACP with the enzyme, *enoyl-ACP reductase* yields butyryl-SACP.

The butyryl-ACP is then recycled and then condensed with another malonyl ACP to

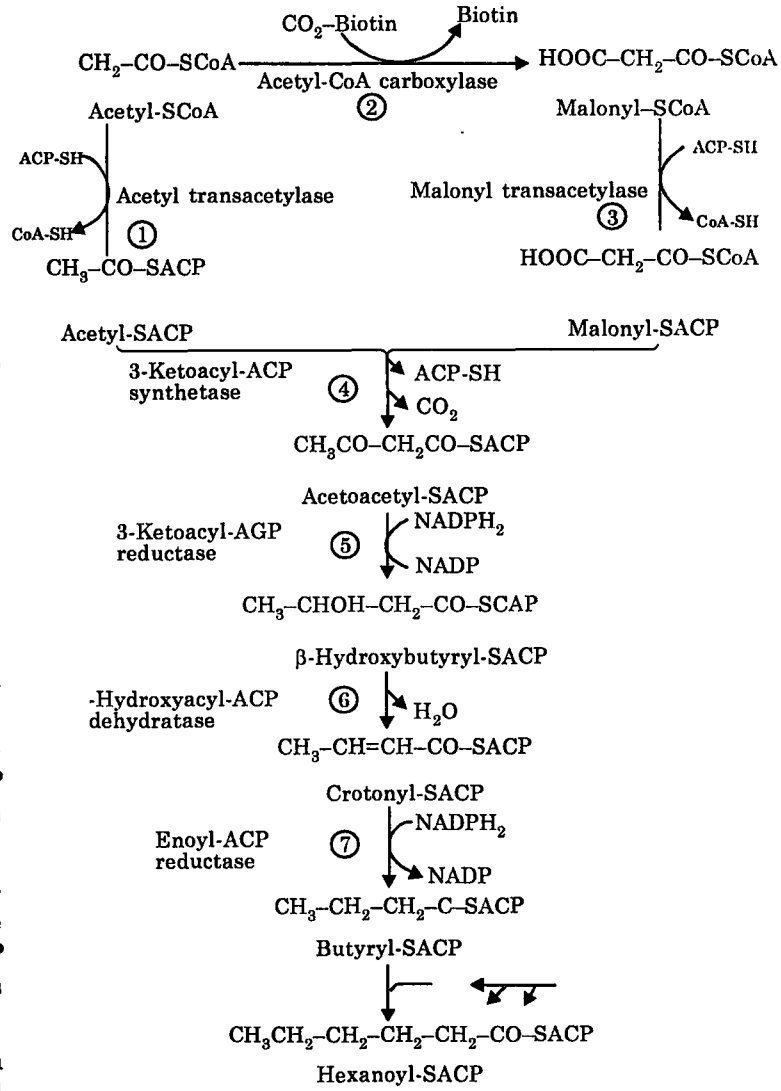


Fig. 21.5. Malonyl pathway of fatty acid biosynthesis.

form a 6-carbon fragment. The essential steps involved in fatty acid biosynthesis via the malonyl pathway are given in Fig. 21.5.

2. Biosynthesis of unsaturated fatty acids : Basically, two distinct mechanisms for the synthesis of unsaturated fatty acids in micro-organisms are known which are : (1) dehydrogenation of long-chain saturated fatty acids and (2) elongation of already unsaturated short-chain intermediates. The first is termed as the aerobic system while the second as the anaerobic pathway. The aerobic system is common in eukaryotic organisms and few bacteria, and needs molecular oxygen, $NADPH_2$, ferredoxin, a specific NADPH reductase and the ACP ester of the saturated fatty acid. Bloch has postulated the electron transport chain for oxygen activation in the desaturation reaction as depicted in Fig. 21.6.

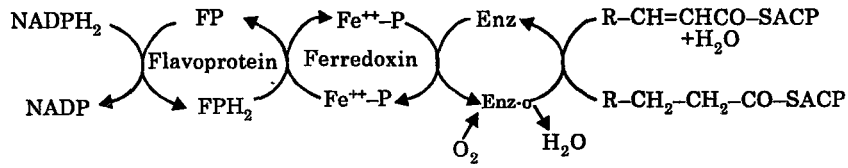


Fig. 21.6. Electron transport chain for oxygen activation in the desaturation reaction.

Typical reactions of this type are given as follows :

$$CH_3(CH_2)_{16}CO\text{—}SACP \xrightarrow[O_2]{NADPH_2} CH_3(CH_2)_7CH{=}CH(CH_2)_7CO\text{—}SACP$$

Stearyl—SACP Oleyl—SACPP

$$CH_3(CH_2)_{14}CO\text{—}SACP \xrightarrow[O_2]{NADPH_2} CH_3(CH_2)_6CH{=}CH(CH_2)_7CO\text{—}SACP$$

Palmitoyl—SACP Palmitoleyl—SACP

It is possible to introduce additional double bonds into the ACP ester of the mono-enoic acid by a similar reaction which gets catalysed by the same enzyme.

The anaerobic pathway is common in some bacteria, especially *E. coli.* In this pathway desaturation occurs at an earlier stage in the synthesis of fatty acids, generally if the chain is 8-12 carbon atoms long. Then, the unsaturated fatty acid chain gets elongated by successive two carbon additions. The sequence starts with the formation of medium chain β-hydroxyacyl-SACP derivative. This derivative gets dehydrated with the elimination of the β-hydroxyl group to form either the *cis*-β-γ or the *trans*-γ-β unsaturated product. The formation of palmitoleic acid and *cis*-vaccenic and from β-hydroxydecanoyl-SACP in *E. coli* is depicted in Fig. 21.7.

The product of the reaction is cis-vaccenic acid (Δ^{11}) but not oleic acid (Δ^9) which is somewhat more common in higher forms. If the same series of reactions starts with β-hydroxydodecanoyl-SACP, the end product will be oleic acid. If this basic pathway and a variety of initial substrates are used, a generalized concept for the synthesis of monounsaturated fatty acids could be formulated (Fig. 21.8). Many of the compounds predicted by this concept have actually been reported to occur in one bacterial species or another.

3. Biosynthesis of glycerol : See Art. 21.3.

21.5 Metabolism of Triglycerides

Triglycerides are glycerol esters of long chain fatty acids. Although bacteria have been widely reported to contain glycerides, the amount of the neutral fats per cell is usually low and mono and diglycerides are reported more often than triglycerides. Glycerides are among the most highly

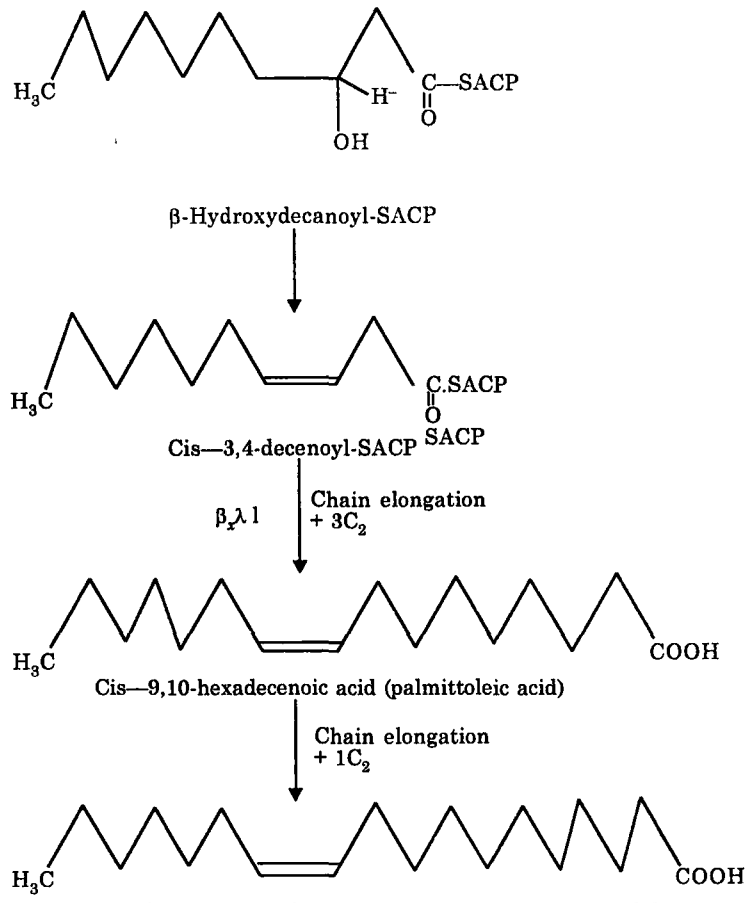


Fig. 21.7. Anaerobic pathway for the synthesis of unsaturated fatty acids.

reduced substances available to micro-organisms. Micro-organisms, however, do not appear to store glycerides as energy reserves. The initial step in the catabolism of glycerides involves hydrolysis to yield *glycerol* and *fatty acids.* This hydrolysis is catalyzed by intracellular and extracellular lipases. Glycerol is then channeled into a variety of glycerol containing lipids.

A. Glycerol Dissimilation

Glycerol is dissimilated by two chemical modes. One begins with *dehydrogenation* followed by *phosphorylation* and the other begins with *phosphorylation* followed by *dehydrogenation.* In both cases, the terminal product is *dihydroxyacetone phosphate* (DHAP). Certain microorganisms are able to utilize not only external glycerol, but also *sn-glycerol-3-phosphate* (G3P) and/or *dihydroxyacetone* (DHA), the intermediates of the two pathways. Metabolic access to all three requires a unique permeation protein. There are considerable variations in the enzyme reactions of the pathways. The kinds of proteins employed by diverse bacterial species for the dissimilation of glycerol, G3P and DHA are enumerated below together with the reactions they catalyze.

1. Glycerol facilitator (or facilitator protein) :

$$\text{Glycerol} \underset{\text{Cell membrane}}{\overset{\text{Equilibration}}{\rightleftharpoons}} \text{Glycerol}$$

The term facilitator implies that the protein catalyzes the equilibrium across the cell membrane, rather than the unhill transport.

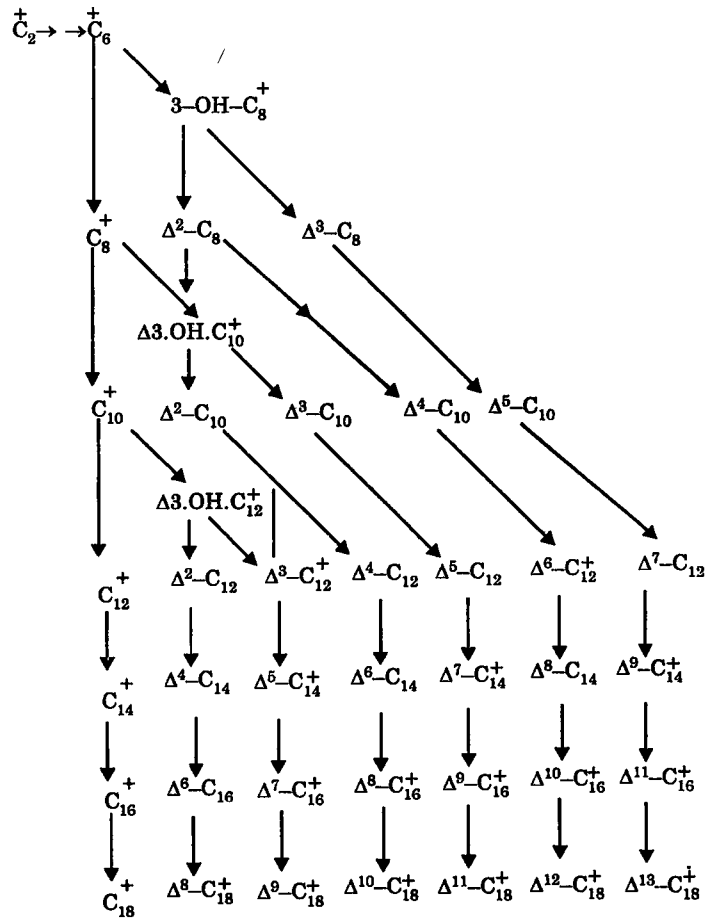


Fig. 21.8. Bacterial synthesis of monounsaturated fatty acids by desaturation and elongation. (+-Compounds detected in bacteria).

2. G3P transport (or permease) :

$$G3P \underset{\text{Cell membrane}}{\overset{\text{Active transport}}{\rightleftharpoons}} G3P$$

3. Glycerol kinase (ATP : glycerol-3-phosphotransferase) :

$$\text{Glycerol} + ATP \longrightarrow G3P + ADP$$

4. DHA kinase :

$$DHA + ATP \longrightarrow DHAP + ADP$$

5. Glycerol dehydrogenase (Glycerol : NAD^+-2 oxidoreductase) :

$$\text{Glycerol} + NAD^+ \rightleftharpoons DHA + NADH + H^+$$

6. Glycerol kinase :

$$\text{Glycerol} + O_2 \xrightarrow{\text{Flavin}} DHA + H_2O_2$$

The existence of this enzyme was postulated in a study of *Gluconobacter oxydans* subsp. *suboxydans,* but has not been characterized *in vitro.*

7. Anaerobic G3P dehydrogenase :

$$G3P \xrightarrow[\text{or nitrate reductase chain)}]{\text{(coupled to fumarate reductase}} DHAP$$

8. Aerobic G3P dehydrogenase :

$$G3P \xrightarrow[\text{or nitrate reductase chain)}]{\text{(coupled to cytochrome oxidase}} DHAP$$

9. G3P oxidase :

$$G3P + O_2 \xrightarrow{FAD} DHAP + H_2O_2$$

10. G3P-synthase (or DHAP reductase) :

$$DHAP + NADPH \rightleftharpoons G3P + NADP^+$$

11. G3P-acyltransferase :

$$G3P + \text{acyl-coenzyme A} \rightleftharpoons \text{Acyl G3P} + \text{coenzyme A}$$

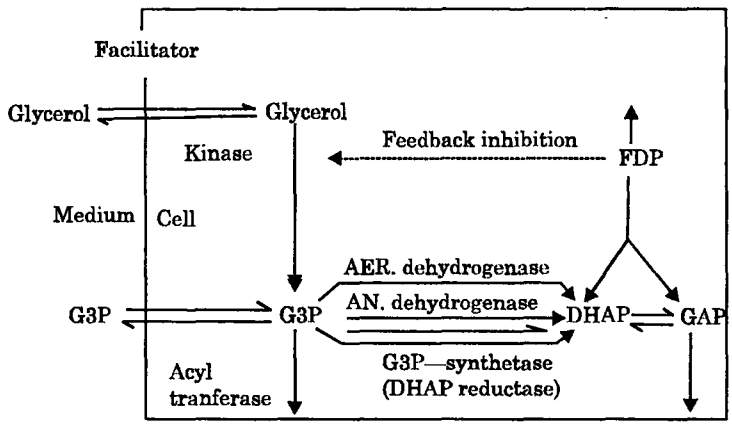


Fig. 21.9. Network for metabolism of glycerol and G3P in *E. coli.*

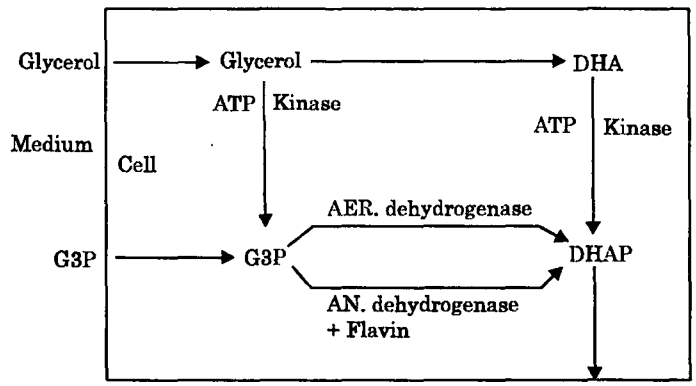
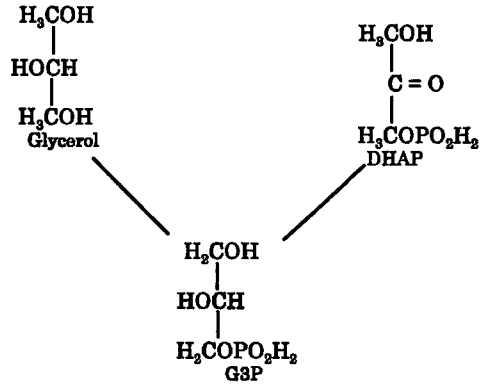


Fig. 21.10. Network for the metabolism of glycerol and GBP in *Klebsiella (Aerobacter) aerogenes.*

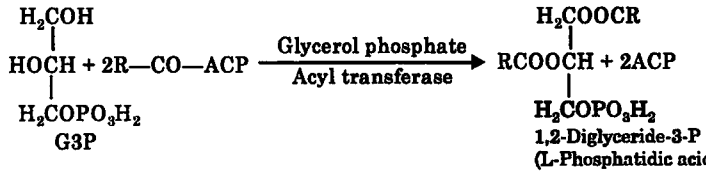
The last two enzymes exert an important influence on the internal pool of G3P. The pathways responsible for growth of *E. coli* and *K. aerogenes* on glycerol and G3P are presented in Fig. 21.9 and 21.10.

B. Synthesis of Triglycerides

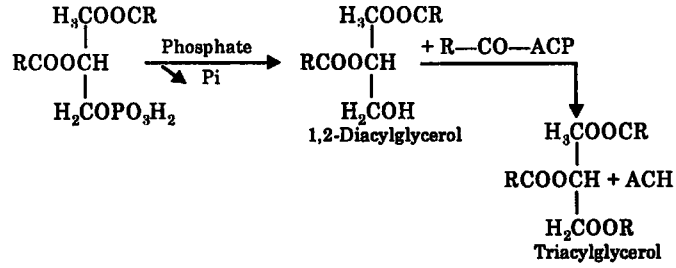
The principal substrates for the synthesis of triglycerides are *glycerol-3-phosphate* (G3P) and acyl-ACP.G3P is formed by phosphorylation of *glycerol* or by reduction of *dihydroxyacetone phosphate* (DHAP).



One mole of G3P is subsequently acylated with 2 moles of fatty acyl ACP to yield 1, 2-diglyceride-3 phosphate termed as phosphatidic acid.



Hydrolysis of *phosphatic acid* by a *phosphatase* yields a *1, 2-diacylglycerol,* which in turn reacts with another mole of acyl-ACP to form a *neutral triglyceride.* Most of the phosphatidic acid, however, is used for the synthesis of phospholipids.



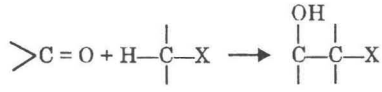
22 Biosynthesis of Alkaloids

22.1 Introduction

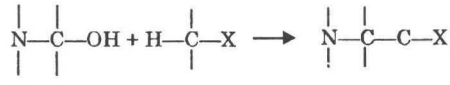
In spite of the large numbers and great diversity of alkaloid structures, it seems possible now to discern a few general principles that are applicable to the biosynthesis of many different alkaloids. Out of the various earlier proposals, the assumptions of Robinson's scheme of biosynthesis are important and are summarised as follows :

(*i*) The fundamental skeletons of alkaloids suffice to form common acids and other small biological molecules.

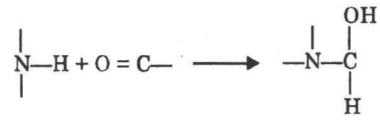
(*ii*) A few simple types of reactions suffice to form complex structures from these starting materials. For instance, the aldol condensation :



the carbinolamine condensation :

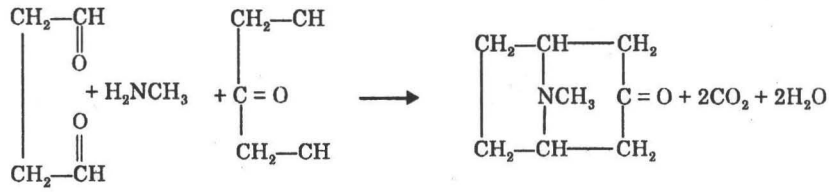


the aldehyde-amine condensation :

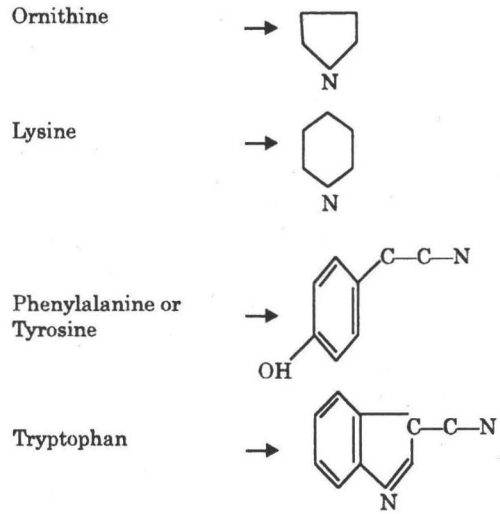


as well as simple dehydrations, oxidations and decarboxylations. In the above reactions, X is representing an activating group like carbonyl.

It is interesting to remark that Robinson's assumptions are never applicable to specific compounds. However, they may be applicable to general groups of structurally related compounds. For example, a possible formation of tropane skeleton from succindialdehyde, methylamine, and acetone dicarboxylic acid might be represented as follows :



It is important to understand that the actual reactions *in vivo* might be resembling closely ornithine, glycine and citric acid, which by undergoing simple reactions could be converted into three represented precursors. Summarising this categorial approach, the names of some common structural elements of alkaloids and the types of precursors from which they might be derived are given below :



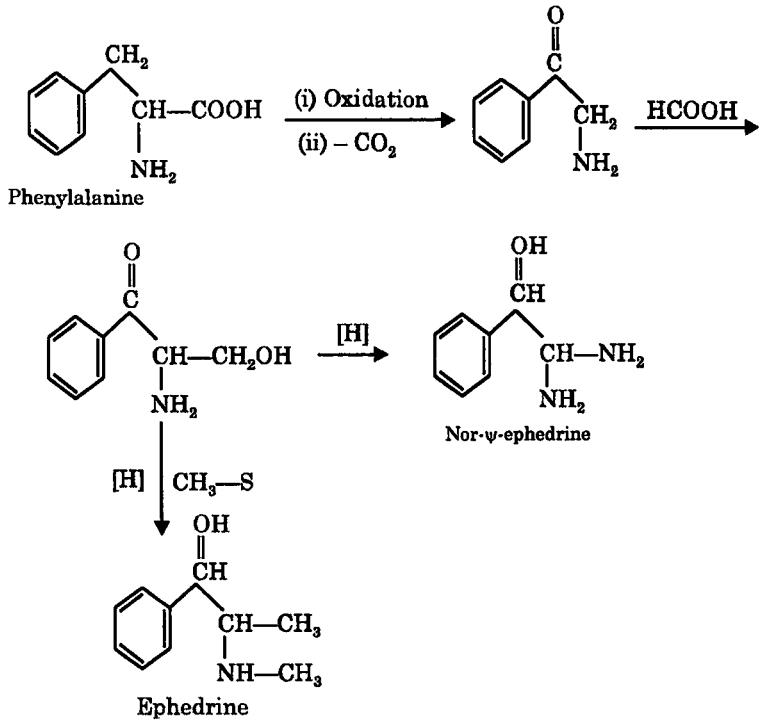
A number of experiments were attempted to demonstrate that with how concentrations and undermild conditions of temperature and pH it becomes possible to carry out reactions of the hypothetical precursors to form complex structures which are resembling alkaloids.

Biochemical experiments have given support to the assumptions of Sir Robert Robinson although certain discrepancies and variations have been reported.

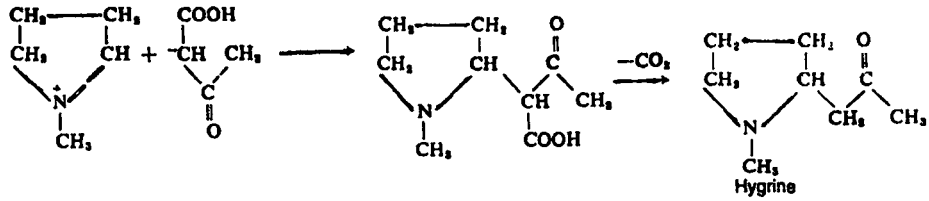
We shall now discuss the hiosynthesis of various groups of alkaloids.

1. Phenylethylamine group : According to Robinson, the precursors for the biosynthesis of alkaloids of phenylethylamine and isoquinolide groups are the amino acids like phenylalanine, tyrosine and 3, 4-dihydroxyphenylalanine. Leete in 1952 proved Robinson's view by using labelled

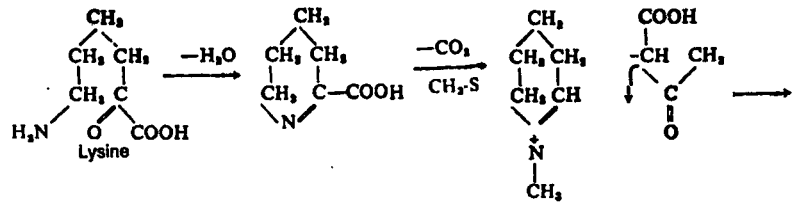
compounds. On the basis of Robinson's view, the biosynthesis of ephedrine may be explained as follows :

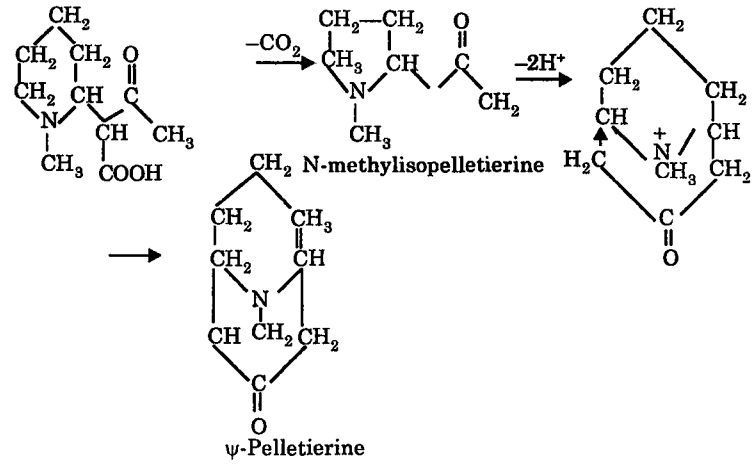


2. Pyrrolidine alkaloids : The precursors for the biosynthesis of pyrrolidine alkaloids are N-methyl-Δ′-pyrrolinium cation and acetoacetic acid. The former is derived from ornithine and related amino acids while the latter from acetic acid. The two precursors react in the following way to form hygrine.



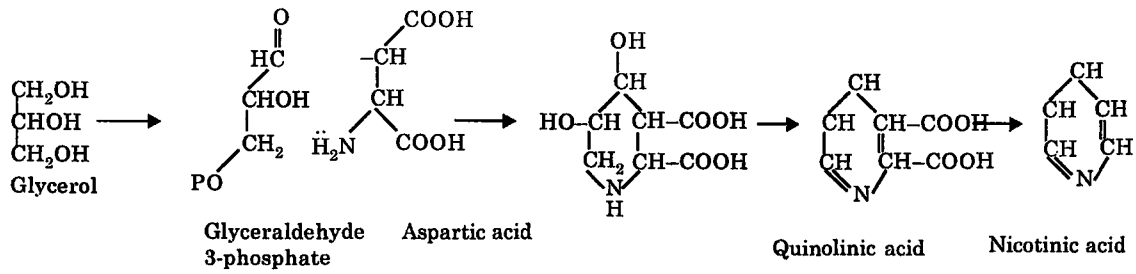
3. Piperidine alkaloids : Several pathways have been reported for the biosynthesis of piperidine alkaloids in the plant. However, the pathway depends upon the nature of the alkaloid. For example, the precursor for the biosynthesis of pelletierines is lysine which is first converted into the N-methyl Δ′-piperidinium cation. The latter then undergoes a Mannich reaction with acetoacetic acid, etc., so as to yield ψ-pelletierine.





4. Pyrrolidine-pyridine alkaloids : An example of these alkaloids is nicotine which consists to two units—pyrrolidine and pyridine.

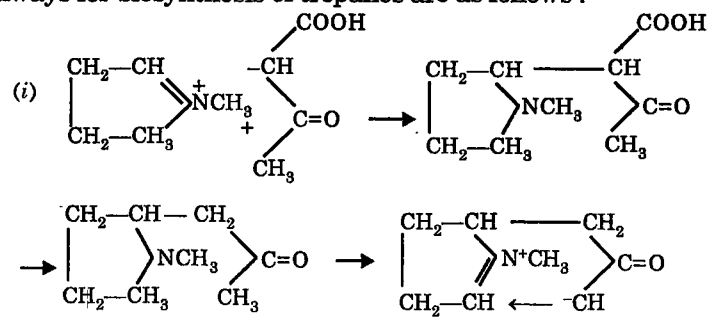
It has been shown by tracer experiments that the precursor of the pyridine ring of nicotine is nicotinic acid. The latter acid, as proved by various experiments, is produced *via* quinolinic acid. It is also proved by various experiments that the biosynthesis of the pyridine ring in nicotinic and quinolinic acids involves glycerol and aspartic acid. One possible biosynthetic pathway is as follows :

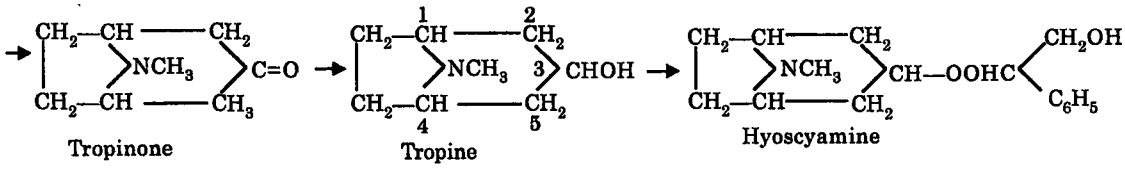


5. Tropane alkaloids : By tracer experiments, it has been proved conclusively that ornithine, N-methylputriscine, hygrine, etc., are the precursors of tropine.

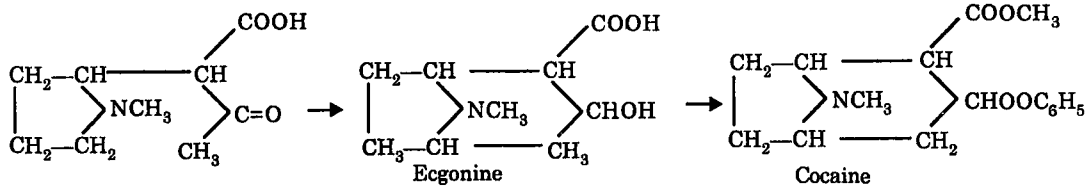
When 2-^{14}C ornithine is used, tropine labelled at C1 is obtained. Further, when N-methylputrescine labelled, $H_2N(CH_2)_4$-$^{15}NH^{14}CH_3$ is used, tropine with $^{15}N^{14}CH_3$ is obtained. This proves the fact that the nitrogen atom in the alkaloid has been derived from the amino acid precursor.

The possible pathways for biosynthesis of tropanes are as follows :



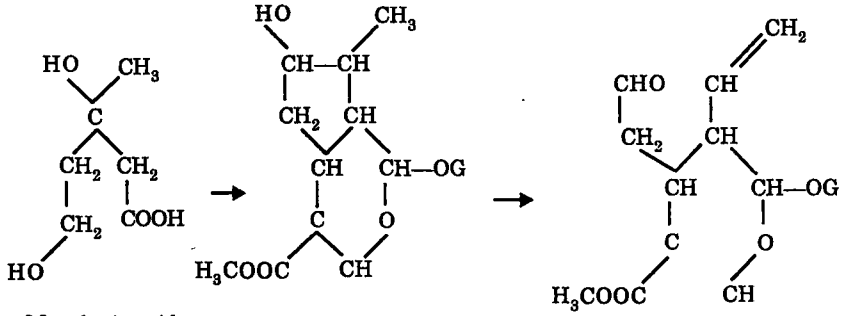


Tropinone Tropine Hyoscyamine



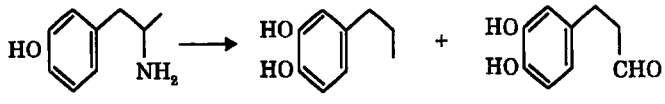
Ecgonine Cocaine

6. Quinoline alkaloids : Tracer experiments have shown that tryptophan is the precursor of biosynthesis of the cinchona alkaloids. Secologanin is another precursor which is derived from loganin, a natural terpenoid of the iridoid group. Secologanin has been derived from mevalonic acid.



Mevalonic acid Loganin Secologanin

7. Isoquinoline alkaloids : (*i*) By tracer experiments, it has been proved that papaverine is obtained from tyrosine. The latter produces a mixture of dopamine and 3, 4-dihydroxyphenylacetaldehyde (or the pyruvic acid).

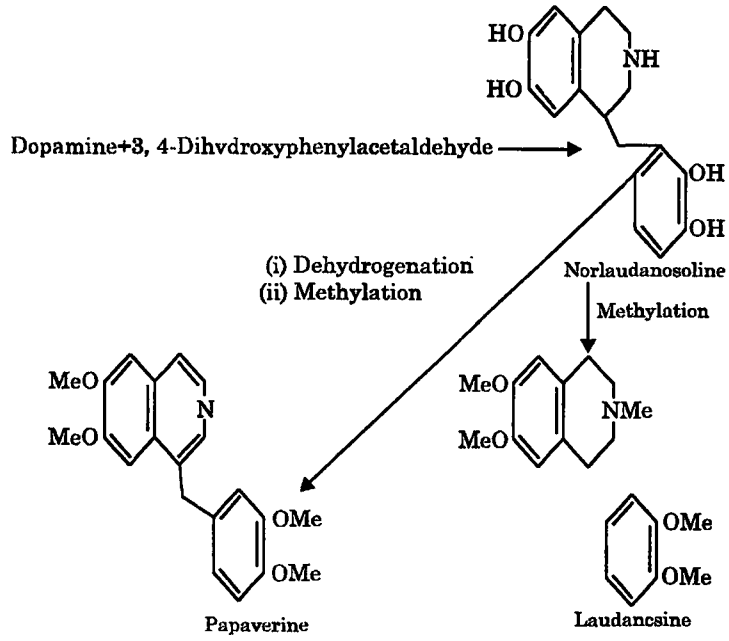


Tryosine Dopomine 3,4-Dihydroxyphenyl-acetaldehyde

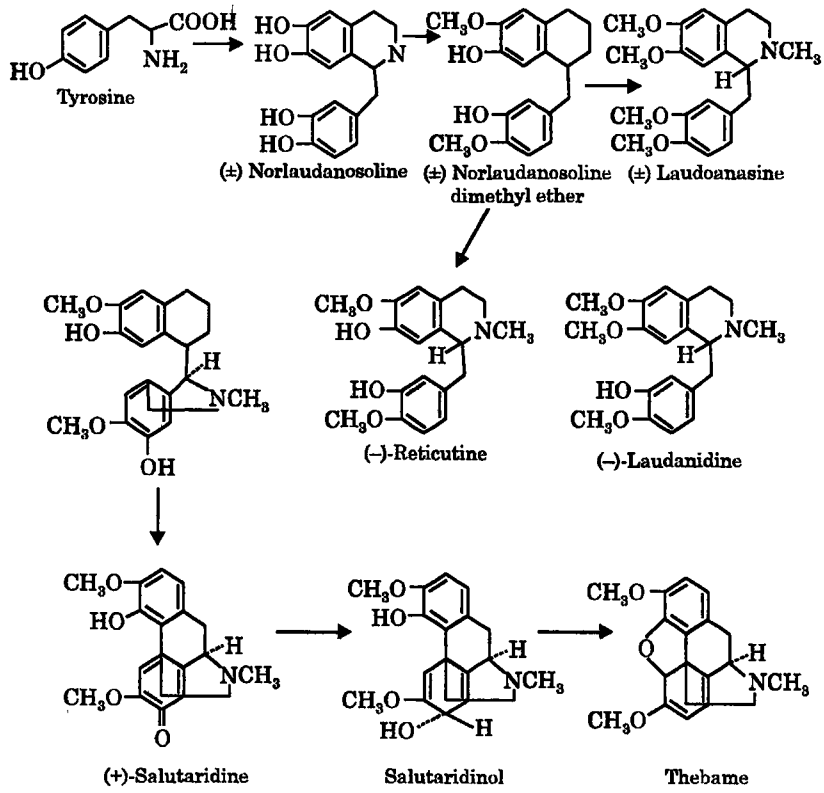
(*ii*) Dopamine and 3, 4-dihydroxyphenylacetaldehyde produced in step (*i*) undergo condensation (Pictet-Spengler), etc., to yield isoquinoline alkaloids.

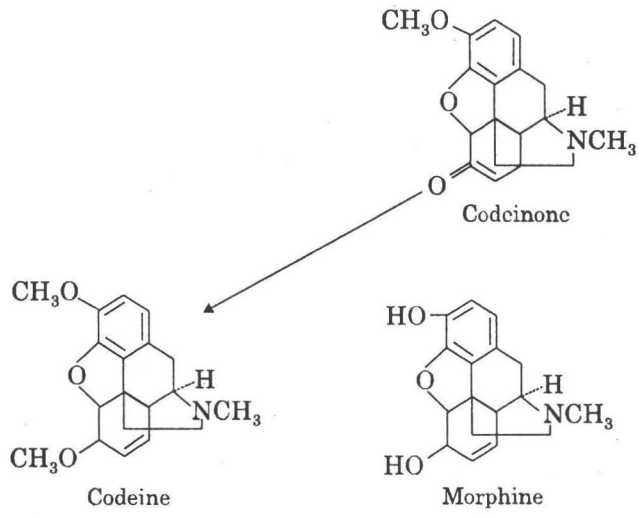
8. Phenanthrene alkaloids (Morphine and Codeine) : Early tracer feeding experiments showed tyrosine-2^{14}C to be precursor of aporphine and morphian alkaloids in poppy seedlings, with the label appearing at C-9 and C-16 of morphine.

More recent experiments have definitely shown that benzylisoquinoline alkaloids like norlaudanosoline, norlaudanosoline dimethyl ether and reticuline (in increasing order of effectiveness) were good precursors of morphian alkaloids when injected into capsules of P *somniferum*. The rate of incorporation of radioactivity into the alkaloids in plants growing in an atmosphere containing $^{14}CO_2$ and the rate of incorporation of activity from labelled tyrosine have been measured, and in both cases the activity has been found first in thebaine, then in codeine and finally in morphine, indicating that this is the order in which the bases are formed in the plant.

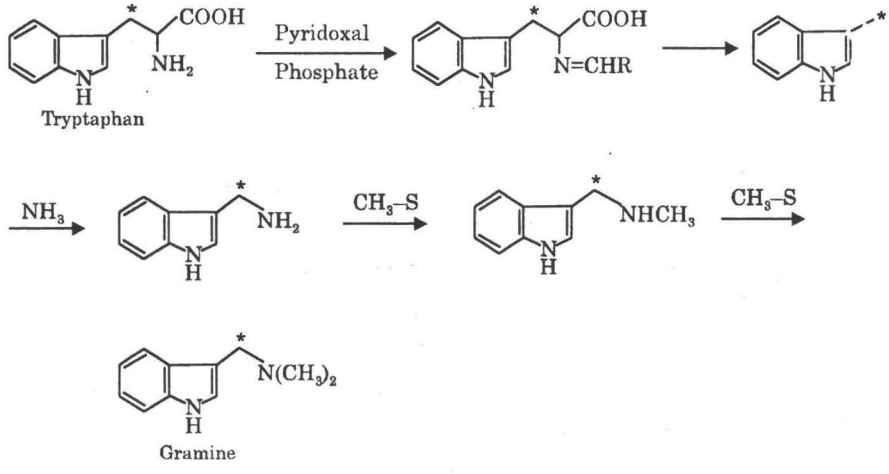


The pathways of morphine alkaloid biosynthesis are shown as follows :





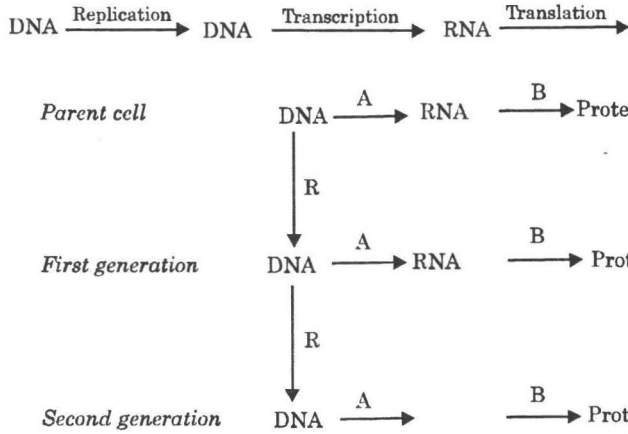
9. Indole alkaloids : For the biosynthesis of most of the indole alkaloids, the precursor is tryptophan. For example, gramine is obtained from tryptophan. In the scheme given below, the labelled β-carbon atom of the side-chain (with tritium) in tryptophan remains retained in gramine.



23 Protein Synthesis

23.1 Mechanism of Protein Synthesis

Proteins are synthesised in the cytoplasm or ribosomes. The basic mechanism of protein synthesis is that DNA makes RNA, which in turn makes protein. The central dogma of protein synthesis is expressed as follows :



The step A is called *transcription step*, step B *translation step* and step R, *self-replication step*. Thus, protein synthesis consists of two main events :

1. Transcription
2. Translation

Both these steps have been depicted in Fig. 23.1. We shall discuss these steps one by one.

23.2 Transcription

Introduction : The copying of a complementary messenger RNA strand on a DNA strand is called transcription. The DNA strand unwinds and one of the two strands forms a mRNA strand. The nucleotides of mRNA are complementary to those of the DNA strand. However, in mRNA in the nuclear membrane to the cytoplasm here it forms a complex with a group of ribosomes (*polyribosome*).

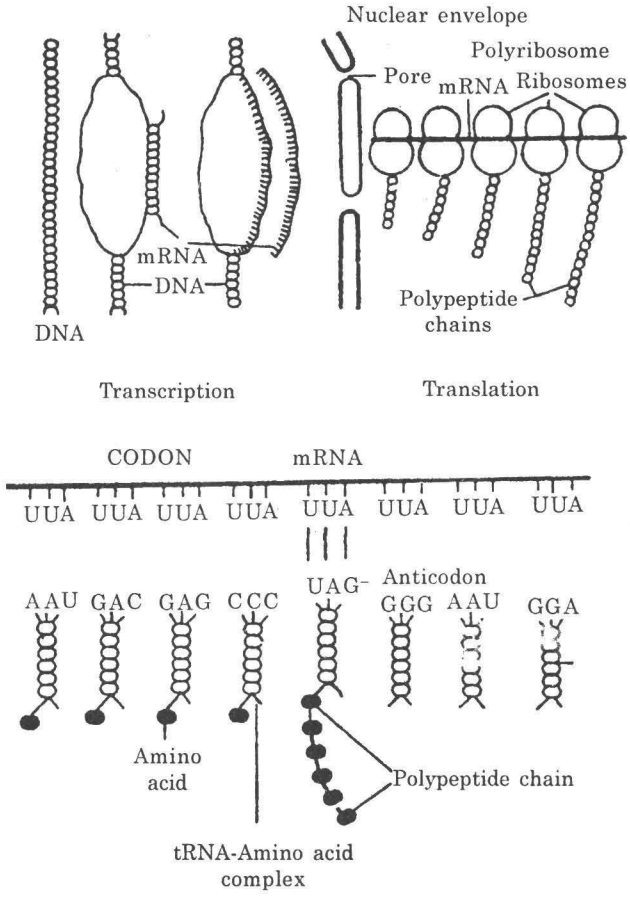


Fig. 23.1. General scheme of protein synthesis.

Components of the Transcription Machinery : Transcription requires a *template,* activated precursors, a *divalent metal ion,* and *RNA polymerase.* .

(i) Template : The preferred template for transcription is double, stranded DNA (dsDNA). Of the two strands, however, only one strand, called the "sense" strand, transcribes mRNA. Single-strand DNA (ssDNA) can also serve as a template.

(ii) Activated precursors : Synthesis of the mRNA strand requires all four ribonucleoside triphosphate—ATP, GTP, UTP and CTP.

Adenine-ribose-triphosphate (ATP)

Guanine-ribose-triphosphate (GTP)

Uracil-ribose-triphosphate (UTP)

Cytosine-ribose-triphosphate (CTP)

(iii) Diyalent metal ions : Mg^{2+} or Mn^{2+}, are effective in transcription. *In vivo,* synthesis requires Mg^{2+}.

(iv) RNA polymerase : RNA polymerase is shown in Fig. 23.2. It consists of a core enzyme with subunits (α, α, β, β, ω) and a *sigma* (σ) factor. The sigma factor initiates transcription of mRNA on the DNA template and the core enzyme continues transcription.

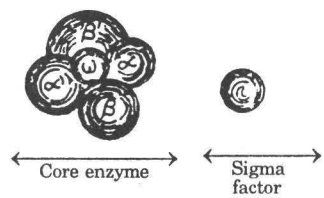


Fig. 23.2. Schematic representation of the enzyme RNA polymerase.

Various Steps of Transcriptions

These are as follows :

1. Initiation of transcription : It involves the following steps :

(i) Recognition, binding and initiation sites on DNA : (Fig. 23.3). RNA polymerase initially binds to an RNA polymerase *recognition site* on the DNA promoter region. It then diffuses to a *binding site* consisting of a sequence of seven bases, which never differs by more than two bases from the following sequence : 5′ TATPuATG. The start of mRNA transcription takes place at the *initiation site.* This consists of one of two bases near the binding site (6 or 7 bases away).

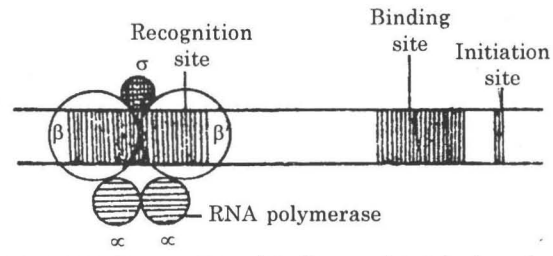


Fig. 23.3. Recognition, binding and initiation sites on DNA.

(ii) Cryptic initiation sites in eukaryotic mRNAs : Prokaryotic mRNAs are *polycistronic* and can initiate protein synthesis at internal positions. On the other hand eukaryote mRNAs are *monocistronic.* They have been described as containing only one initiation site for polypeptide chain by Jacobson and Baltimore (1968) who suggested that in animal cells a single mRNA produces a single polypeptide chain (one-mRNA, one-polypeptide hypothesis). (In some cases a *polyprotein* is produced, which later undergoes cleavage to form several proteins).

Although eukarytote mRNA can have internal polypeptide initiation sites, only the site nearer to the 5′ end is active. Thus, the general rule of Jacobson and Baltimore that one mRNA directs the synthesis of only one polypetide chain is still valid. However, in view of the discovery of internal initation sites the rule is better expressed as follows : "one mRNA has only one active initiation site and the active site is always that which is nearest to the 5′ end" (Smith, 1978).

(iii) Unwinding of DNA double helix : The binding of RNA polymerase to DNA results in local unwinding of the DNA double helix. A short segment of DNA molecule opens up. Synthesis of mRNA now begins on one of the two strands of dsDNA.

(iv) RNA chains start with pppG or pppA : The first base of RNA is always purine, either pppG or pppA. In *E. coli* most chains start with pppG while $\phi \times 174$ and T7 bacteriophage chains start with pppA. After synthesis, however, the terminal triphosphate group of nucleotides may be cleaved by endonucleases. This results in RNA chains without terminal triphosphate groups, or chains starting with *pyrimidine* nucleotides.

2. Elongation of RNA chain : Chain elongation takes place by addition of activated ribonucleoside triphosphates (ATP, UTP, GTP and CTP) to one strand of the DNA template. For each necleotide added to the growing RNA chain a pyrophosphate (PPi) is given off. This is rapidly hydrolysed to inorganic phosphate (Pi). The synthesis of the RNA chain is energised by expenditure of energy in the form of pyrophosphates. For each necleotide monomer added to the chain two high energy phosphates are expended. The entire reaction may be summarized as follow :

$$\underset{\text{template}}{\text{DNA}} + \text{Activated ribonucleoside triphosphates (ATP+GTP CTP+UTP)} \xrightarrow[\text{polymerase}]{\text{RNA}} \underset{\text{template}}{\text{DNA}} + \text{RNA polymer+P~P (AMP, GMP CMP, UMP)}_{4n}$$

Elongation of the RNA chain takes place by means of the core enzyme which moves along the DNA template.

Some facts about elongation of RNA chain are as follows :

(i) Dissociation of the sigma factor : After commencement of chain elongation the sigma factor dissociates from the core enzyme. It can now be used by another polymerase molecule.

(ii) RNA chains grow in the 5'-3' direction : If RNA chains are synthesized in the 5'-3' direction, then the first necleotide should have a triphosphate group (P~P~P~). If, on the other hand, the chain grows in the three 3'-5' direction then the triphosphate group would be on the necleotide at the growing end. It has been found that the triphosphate group is attached to the first necleotide. This shows that chain growth takes place in the 5'-3' direction. During chain elongation the region of DNA that had been transcribed regains its double helical conformation as the next region of DNA unwinds. The newly formed RNA chain has a triphosphate group at its 5' end and a free hydroxyl group at the 3' end (Fig. 23.4).

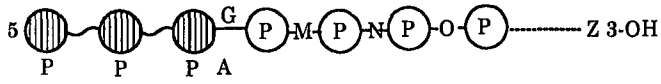


Fig. 23.4. Newly formed RNA chain.

(iii) Only one DNA strand of a gene transcribes mRNA : In double stranded DNA a given gene is transcribed from only one of the two strands. The transcribed RNA is complementary to only one of the two strands. All the transcribing genes need not, however, be on one strand of the DNA double helix. One gene may transcribe *m*RNA from one strand while another transcribes from the other strand. When both strands of the DNA double helix transcribe mRNA, the transcription is said to be *symmetrical* (Fig. 23.5). In a circular bacterial chromosome this would mean clockwise transcription on one strand and counterclockwise transcription on the other. In some cases RNA may be transcribed exclusively by one strand. Such transcription is said to be *asymmetrical.*

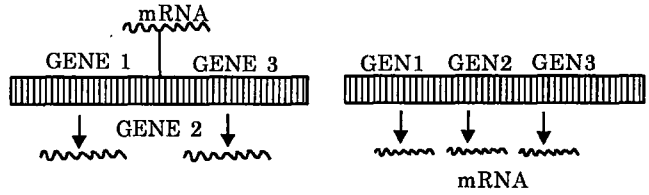


Fig. 23.5. Symmetrical and asymmetrical transcription.

RNA Chain Termination : Termination of RNA chain synthesis appears to be brought about by two types of mechanisms. In the *first type* the termination signal appears to be recognized by DNA itself. RNA polymerase reads an extended poly (A) sequence on DNA. This results in an RNA transcript with a terminal poly (U) sequence.

The *second type* of termination signal involves an additional protein called the *rho* (ρ) *factor*. This is a tetramer of molecular weight 200,000. The rho factor probably binds to RNA polymerase. It is, however, not certain whether it also, or exclusively, binds to DNA.

Richardson (1978) has proposed a model for rho function (Fig. 23.6). The rho factor catalyses the reaction :

$$ATP/GTP/CTP/UTP + H_2O \xrightarrow{RNA, MG^{2+}} ADP/GDP/CDP/UDP + Pi$$

It has been suggested that rho recognizes in RNA a site rich in pyrimidines and containing at least a few *cytidylate residues.* The requirement for magnesium ions is not absolute.

NTPase activity of rho is essential for termination. NTPase activity is in turn dependent on RNA. Thus the requirement for RNA in termination is absolute.

Not all RNA is copied along DNA templates. Certain viruses, *e.g.*, the tobacco mosaic virus (TMV) contain RNA as the genetic material instead of DNA. This RNA can serve directly as *m*RNA. A complementary RNA strand can also be synthesized by a RNA *primer*.

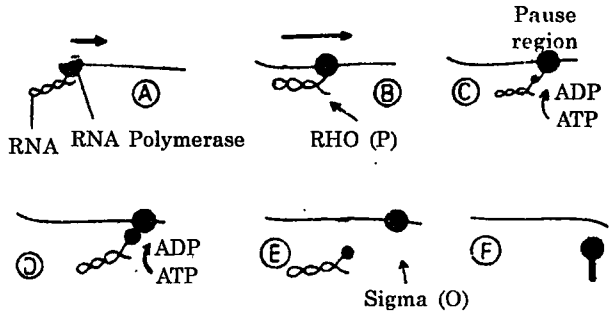


Fig. 23.6. Richardson's (1978) model of RNA chain termination by rho factor.

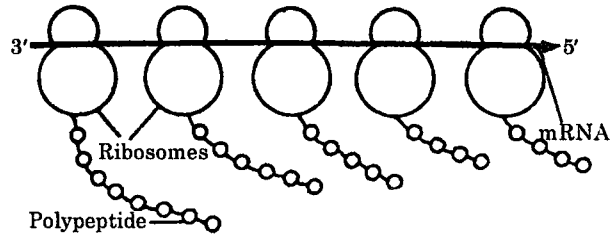


Fig. 23.7. Formation of polypeptide chains on a polyribosome.

Messenger RNA functions in transcribing information from the DNA molecule and carrying to the ribosomes where it is translated into protein. It is, therefore, the carrier of information for protein synthesis from the genes to the sites of protein synthesis. Each mRNA strand becomes associated with the smaller subunits of a group of ribosomes to form a *polyribosome* or *polysome* (Fig. 23.8). These may be found free in the cytoplasm, as in bacteria, or may be associated with the endoplasmic reticulum, as in higher cells. Bacterial ribosomes contain two subunits, 30S and the 50S subunits. The 30S subunit alone can bind *m*RNA. In animal cells the *m*RNA binds to the 40S subunit.

The step-by-step enlongation of the RNA chain on a DNA template is catalysed by the enzyme RNA polymerase. RNA polymerase was isolated from *E. coli* by Chamber and Berg (1962).

The above described scheme of RNA synthesis is depicted in Fig. 23.9.

23.3 Translation

During translation the genetic information present in *m*RNA directs the order of specific amino acids to form a polypeptide or protein.

The *m*RNA has a series of triplet bases, each triplet forming *codon*. The codons pair with *anticodons* of the *t*RNA molecule. Each anticodon consists of three free bases. The pairing follows the A-U and G.C.

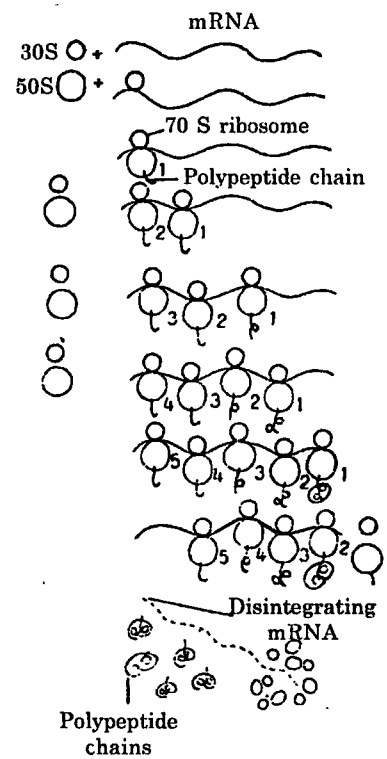


Fig. 23.8. Formation of polyribosomes during protein synthesis.

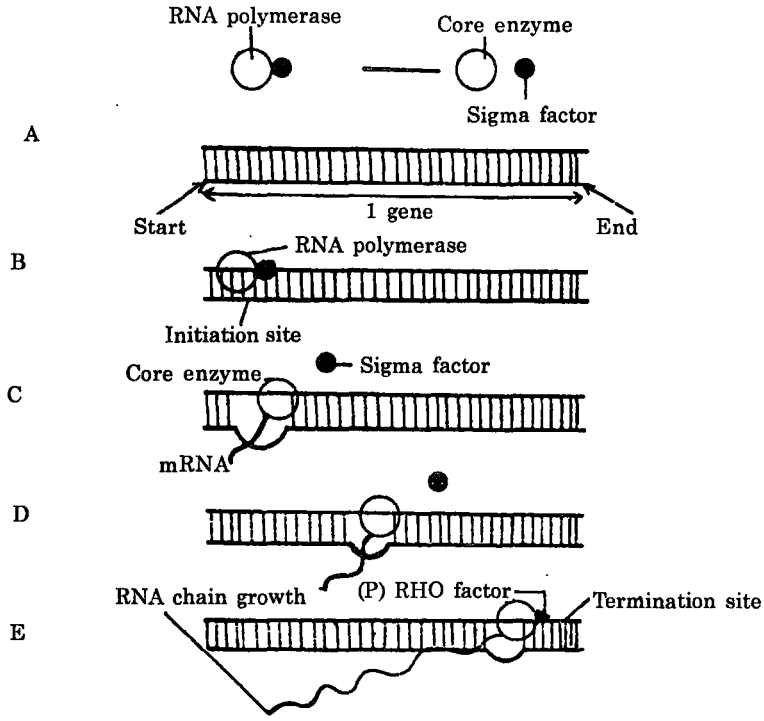


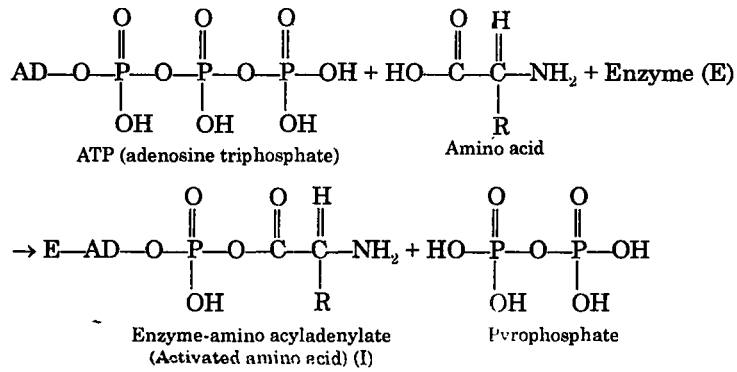
Fig. 23.9. Scheme of RNA synthesis.

A. Sigma factors and core enzyme join to form RNA polymerase.
B. RNA polymerase attaches to initiation site.
C. DNA unwinds and core enzyme catalyses synthesis of mRNA. Sigma factor dissociates from core enzyme.
D. RNA chain grows longer.
E. Termination of chain growth at termination site by rho factor.

combination. Thus the codon GUC pairs with the anticodon CAG of tRNA. Thus the series of codons on mRNA determines the series of anticodons of the different tRNA molecules, and hence of the amino acids. Since the triplets of mRNA in turn depend upon the series of bases in DNA, it follows that the DNA molecule determines the sequence of amino acids, and hence the structure of the protein molecule.

The translation involves the following steps :

1. Activation of amino acids : The 20 amino acids (which are building blocks of proteins) are activated by ATP in the presence of specific activating enzyme (E) also called the *aminoacyl synthetases* to form enzyme-aminoacyl adenylates (also called aminoacyl AMP). In this process, pyrophosphates are released.



The activation of amino acids is shown in Fig. 23.10.

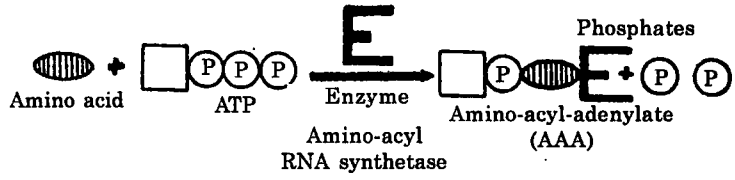


Fig. 23.10. Activation of amino acid.

The activation of amino acids by activating enzymes has a large degree of specificity, each amino acid being activated by a specific enzyme. However, some activating enzymes can activate more than one amino acid, *e.g., isoleucin-tRNA synthetase* can also activate *valine.*

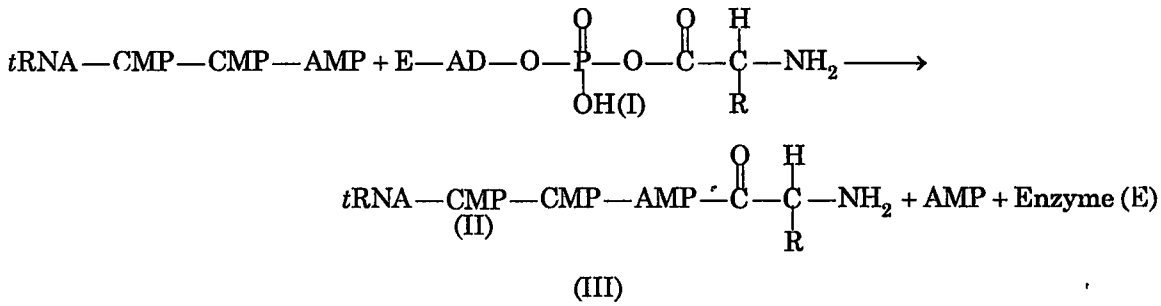
2. Addition of nucleotides to transfer RNA : In order to introduce two cytidine monophosphate groups (AMP) to its end, transfer RNA reacts with two molecules of CTP, cytidine triphosphate. The resulting compound reacts with ATP to add adenosine monophosphate to CMP group. All these reactions are catalysed enzymatically. Only in this condition, the transfer RNA is ready to accept an amino acid.

$$t\text{RNA} + \text{CTP} \underset{}{\overset{\text{Enzyme}}{\rightleftharpoons}} t\text{RNA—CMP} + \text{Pyrophosphate}$$

$$t\text{RNA—CMP} + \text{CTP} \overset{\text{Enzyme}}{\rightleftharpoons} t\text{RNA—CMP—CMP} + \text{Pyrophosphate}$$

$$t\text{RNA—CMP—MP} + \text{ATP} \overset{\text{Enzyme}}{\rightleftharpoons} t\text{RNA—CMP—CMP—AMP} + (\text{Pyrophosphate}) \text{ (II)}$$

3. Transference of amino acid from enzyme—aminoacyl adenylate complex to transfer RNA : The compound (II) reacts with (I) to form transfer complex, *t*RNA—CMP—CMP—AMP—amino acid (III).



(III)

4. Transference of amino acids from transfer RNA to ribosomes and formation of a polypeptide chain : Now the *t*RNA molecules will transfer their amino acids to ribosomal RNA. The latter has a coding system which will determine the sequence of amino acids attached to it. For this reaction, the presence of GTP (guanosine triphosphate) is required.

How the amino acids are joined together in the specific sequence. This sequence will depend upon base pairing between the triplet sequence (codons) on the *m*RNA, and complementary triplet sequences (anticodons) on the (III). This interaction is taking place on the surface of ribosome. Thus, by base-pairing mechanisms, it becomes possible to bring amino acids attached to (III) into the specific (correct) sequence for the assembly of a polypeptide in which the peptide bonds are formed by the nucleophilic attack by the $-NH_2$ group of one amino acid residue on the activated carboxyl group of the preceding amino acid. After the discharge of amino acids, the molecule (III)

becomes separated from the *m*RNA and then this will be used further. Hence the synthesis of a polypeptide chain takes place in a step-wise manner from the N terminal end and will proceed linearly towards the free end with the free carboxylic group (Fig. 23.11).

5. Release of protein from RNA molecule : As there occurs the movement of one ribosome along the *m*RNA away from the starting point, it become possible for a second ribosomal particle to undergo combination at the end of *m*RNA and also starts synthesising the peptide chain. Hence, a polypeptide chain is synthesised at each ribosome. Actually, a large number of ribosomes are attached along a messenger RNA. Each of these ribosomes is synthesising protein and moving towards the end of the messenger RNA. Such a *m*RNA-multiribosomal complex system is called a polysome system.

Now as the ribosome is moving along the *m*RNA chain, there occurs the increase in length of the polypeptide chain and as it reaches the end of mRNA chain, the polypeptide chain releases into the cytoplasm where it undergoes folding to yield secondary and tertiary structures. Finally, the ribosome is returning to the other end of mRNA chain to restart the whole operation as described above (Fig. 23.12).

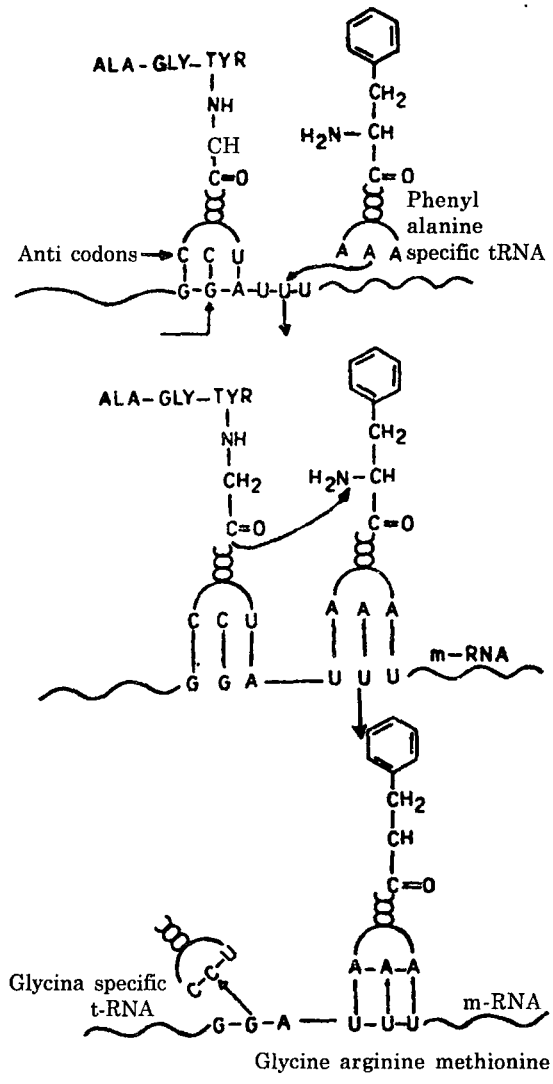


Fig. 23.11. Growth of a peptide chain.

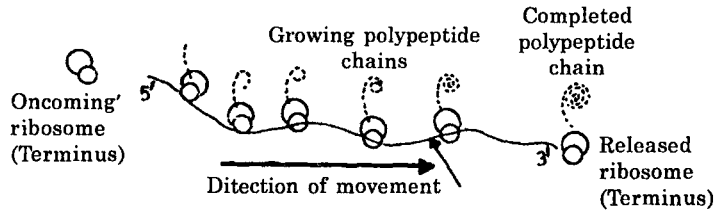


Fig. 23.12. A polysome system.

Role of the different types of RNA in protein sysnthesis is depicted in Fig. 23.13.

23.4 Role of Ribosomes

Protein synthesis takes place only on the surface of ribosomes. Ribosomes are small cellular particles. These are composed of RNA, proteins and certain divalent metallic ions. There are about 15000 ribosomes in rapidly growing *E. coli* cell.

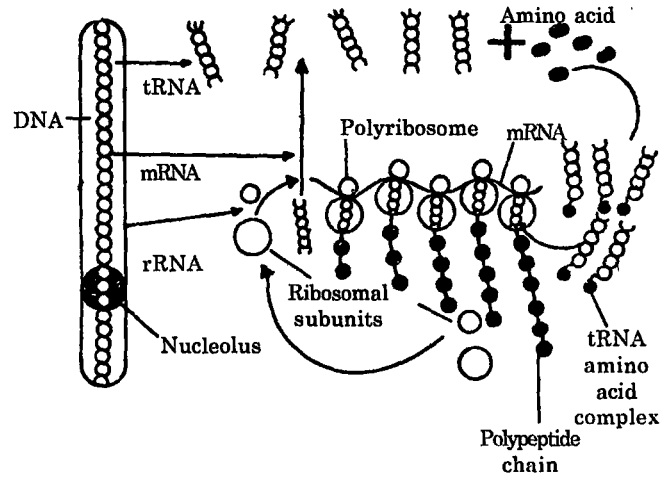


Fig. 23.13. Role of the different types of RNA in protein synthesis.

Ribosomes are of two basic types, 70S and 80S ribosomes. The 'S' refers to Svedberg units. This is a sedimentation coefficient which shows how fast a cell organelle sediments in an ultracentrifuge. The heavier a structure the more is its sediments in an ultracentrifuge. The heavier a structure the more is its sediments in an ultracentrifuge. The heavier a structure the more is its sedimentation coefficient. Sedimentation coefficients are not additive. Thus the 30S and 50S subunits of a ribosome together make up a ribosome with a sedimentation coefficient of 70S and not 80S.

80S ribosomes are found in *eukaryotes* (organisms whose cells have true nuclei bounded by nuclear envelopes) *e.g.*, algae, fungi, higher plants and animals (Fig. 23.14). The 80S ribosome of animals consist of a large 60S subunit and a small 40S subunit.

70S ribosomes are relatively smaller and are found in *prokaryotes* (organisms whose DNA is not bounded by a nuclear envelope), *e.g.*, bacteria. The 70S ribosome consists of a large 50S subunit and a small 30S subunit. The RNA protein ratio is 2:1 in the 70S ribosome while in the 80S ribosome it is 1:1.

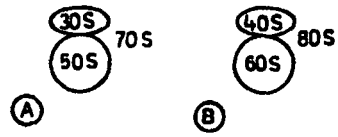


Fig. 23.14. Diagrams of
(A) 70S ribosme of prokaryotes and
(B) 80S ribosome of eukaryotes.

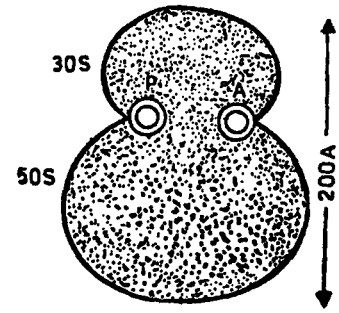


Fig. 23.15. A bacterial ribosome. Ribosomes are the site of protein synthesis.

All ribosomes are made up of two sub-units (Fig. 23.15), the larger subunit is about twice the size of the smaller unit. Each ribosome is having two holes, the peptidyl (p) and the amino acyl (A). Both of these holes are made partly of smaller unit and partly of larger sub-unit.

Functions of ribosomes : Ribosomes take part in protein synthesis. Two or more ribosomes simultaneously engaged in protein synthesis on the same mRNA strand form *polyribosomes.*

The ribosome functions as a template, bringing together different components involved in the synthesis of proteins. Interaction of the tRNA-amino acid complex with *m*RNA, which brings about translation of the genetic code, is coordinated by the ribosomes.

Ribosomes also have a protective function. The *m*RNA strand which passes between the two subunits of the ribosome is protected from the action of the enzymes of the nucleus (*nucleases*). Similarly the nascent polypeptide chains passing through the tunnel or channel between the subunits are protected against the action of protein digesting enzymes.

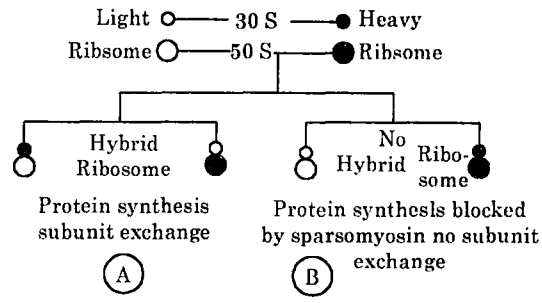


Fig. 23.16. Experiment with 'light' and 'heavy *E. coli* ribosomes to show that (A) subunit exchange takes place during protein synthesis and (B) when protein synthesis is blocked by sparsomycin there is no submit exchange.

Dissociation and reformation of ribosomal subunits take place at some stage in protein synthesis. This is shown by experiments with *E. coli* (Fig. 23.16). Ribosomes made '*heavy*' by isotopes were mixed with '*light*' ribosomes. When protein synthesis occurred some *hybrid* ribosomes were formed, which had one heavy and one light subunit. If after mixing light and heavy ribosomes, protein synthesis was blocked by *sparsomycin,* no hybrid ribosomes were formed. This shows that exchange of subunits takes place only during protein synthesis.

23.5 Regulation of Protein Synthesis : Operon

The total amount of DNA present in a cell may contain from a few to thousands of genes, although the different types of cells in the body of a multicellular organism differ in structure and function, their genes are identical, since all the cells are ultimately derived from the zygote.

As development proceeds certain genes become active while others became inactive, *i.e.,* the genes are "switched on" and "switched off" at different times. This process is called *differential gene action.* When genes are active they direct the formation of enzymes which affect certain traits. The metabolic products formed may repress synthesis of enzymes (*feed-back or end product inhibition*). Thus, enzyme synthesis is induced and repressed at different time.

A hypothesis to explain induction and repression of enzyme synthesis was first put forward by Francois Jacob and Jacques Monod (1961) of the Institute Pasteur in Paris. The scheme proposed by these workers is called the *operon model.* The operon consists of the following components :

(*i*) Structural genes
(*ii*) Operator genes
(*iii*) Promoter genes
(*iv*) Regulator genes

Let us discuss these one by one.

(i) The structural genes : The structural genes direct synthesis of cellular proteins through messenger RNA (*m*RNA) and determine the sequence of amino acids in the proteins synthesized. There are as many structural genes in an operon as there are proteins of polypeptides under common control. Each structural gene may be controlled independently and transcribes a separate *m*RNA molecule, or all the structural genes of an operon may form one long *polycistronic or polygenic m*RNA molecule. Polygenic *m*RNAs are common in bacteria and bacterio-phages. All known eukaryotes, however, contain only a single functional site for initiating protein synthesis.

(ii) The operator gene : The operator gene is adjacent to the first structural gene and controls the structural genes. It determines where or not the structural genes are to be repressed by the *repressor,* a product of the regulator gene. The *operator* is recognized by the *repressor* protein, which binds to the operator, forming an *operator-repressor* complex.

(iii) The promoter gene : This gene is continuous with the operator gene.

(iv) The regulator gene : The regulator gene directs the synthesis of a protein, which may be an *active repressor* or an *inactive repressor* (*aporepressor*). In the *inducible system* the active repressor formed has an affinity for the operator gene. In the absence of an inducer (Fig. 23.17, 23.18, A, B and 23.19) the *lac* repressor protein binds to the operator gene and blocks the path of RNA, polymerase. Thus the structural genes are unable to transcribe *m*RNA, and protein synthesis does not take place. In the presence of an *inducer,* (*e.g., lactose*) the repressor protein binds to the inducer to form an *inducer repressor, complex* (Fig. 23.19). Each repressor subunit has one inding site for the inducer. The repressor undergoes a conformational change which makes it inactive. It cannot now bind to the operator gene, and hence the structural genes can synthesisze proteins.

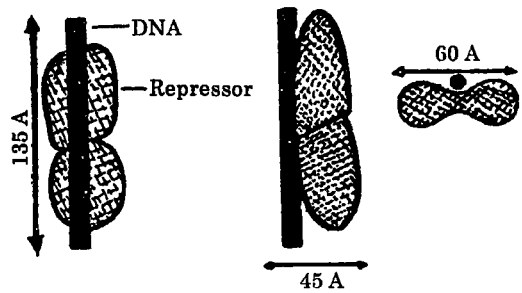


Fig. 23.17. Model of interaction between *lac* repressor and operator : front, side and top views (After Steitz *et al.*, 1974).

In the *repressible system* the repressor protein formed by the regulator gene is inactive and does not block the operator (Fig. 23.19). The structural genes, therefore, synthesize proteins. The repressor is activated in the presence of a *corepressor,* and the *repressor-corepressor* complex blocks the operator gene (Fig. 23.17). The structural genes, therefore, cannot synthesize proteins.

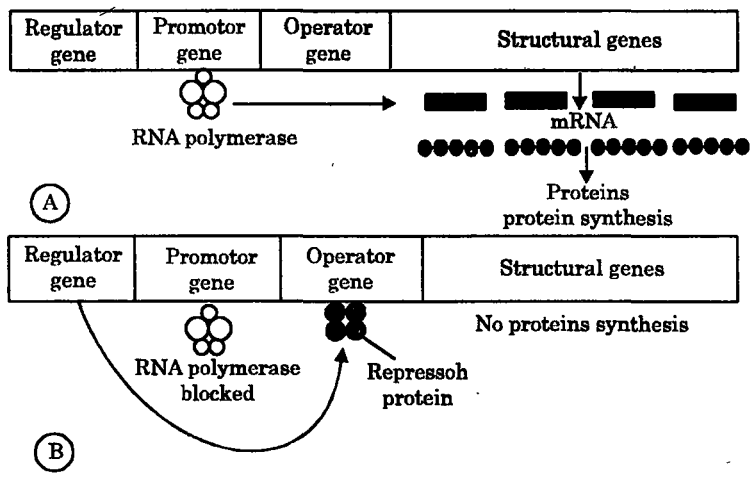


Fig. 23.18. The promoter gene. (A) Binding of RNA polymerase to promoter gene. RNA polymerase moves along the structural genes catalysing synthesis of proteins. (B) Repressor protein formed by the regulator gene binds to the operator gene. Movement of RNA polymerase is blocked, and hence no protein synthesis takes place.

Types of Control Mechanisms

The control of protein synthesis by regulator proteins may be by *induction* or *repression.* There may also be *negative* or *positive control.*

Induction : A category of enzyness called *inducible enzymes* are normally absent from the cell or are present in very small quantities. They increase in quantity only in the presence of an *inducer*. Such a system is called an *inducible system*. A bacterial culture of *E. coli* growing in a medium with glucose as the source of carbon produces only minute quantities of the enzyme β-galactosidase. Only one or two molecules of the enzyme are present. When *lactase* is added to the medium the production of β-galactosidase starts, and within two to three minutes about 3,000 molecules are synthesized by the Z gene, *β-galactosidase* hydrolyses lactose into the sugars galactose and glucose. Some galactose and glucose molecules are converted into *allolactose,* which is the inducer for β-galactosidase synthesis.

In the *lac* system *allolactose* is the *actual inducer* while *lactose* is the *apparent inducer*. An artificial inducer commonly used in experimental work is *isopropylthiogalactoside* (IPTG).

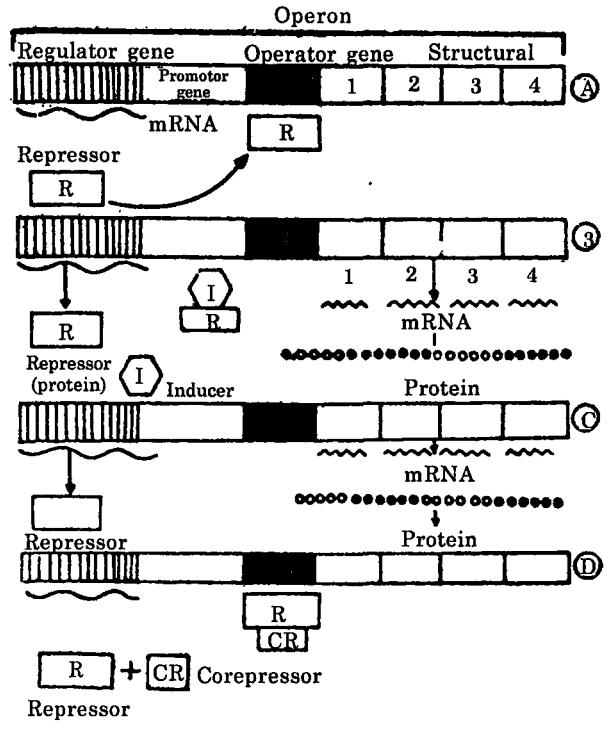


Fig. 23.19. Regulation of protein synthesis. (A) Inducible system, inducer absent. (B) Inducible system, inducer present. (C) Repressible system, corepressor absent. (D) Repressible system corepressor present.

When the *inducer* is *present* the active *repressor* protein produced by the *regulator gene* associates with the *operator gene* and blocks it. No transcription of *m*RNA by the structural genes can therefore take place, and hence there is no enzyme synthesis. Absence of the inducer may be due to the fact that it is not present in the growth medium, or is not synthesized by the microorganism.

When the *inducer* is *present,* the active repressor protein binds to the inducer molecule to form a *repressor-inducer complex* (R-I). A conformational change takes place in the repressor molecule which is inactivated. This prevents binding to the operator, which is therefore not blocked. The structural genes transcribe mRNA, and enzyme synthesis takes place.

Repression : The synthesis of an enzyme may be inhibited by a product of metabolism. In *E. coli* tryptophan synthetase is inhibited by tryptophan. Thus tryptophan synthetase is formed only if the bacterium is grown in a tryptophan free medium. The amino acid *histidine* also acts as a repressor. This process is called *enzyme repression.* In the repressible system the protein produced by the regulator gene is an *inactive repressor,* also called the *aporepressor*. The aporepressor on combining with *a corepressor* or *effector* molecule becomes activated. It undergoes a conformational change which enables it to bind to the operator gene.

When the *corepressor is absent* the inactive aporepressor does not bind to the operator gene. Because the operator gene is not blocked, the structural genes transcribe *m*RNA, and protein synthesis takes place.

When the *corepressor is present* the aporepressor combines with it to form a complex, and is activated. The complex blocks the operator gene. The structural genes are therefore unable to

synthesize mRNA, and consequently no protein synthesis takes place. The corepressor may be a product of one of the enzymes produced by the structural genes.

Table 23.1

REGULATION OF ENZYME SYNTHESIS BY INDUCTION AND REPRESSION

I. Inducible system

1. Inducer absent : Regulator gene ⟶ Active repressor

→ Binds to and blocks operator gene.

→ No *m*RNA transcription by structural genes.

→ No enzyme synthesis.

2. Inducer present : Regulator gene ⟶ Active repressor

→ + inducer ⟶ Inactive repressor

→ No blocking of operator gene

→ mRNA transcription by structural genes

→ Enzyme synthesis.

II. Repressible system

1. A corepressor absent : Regulator gene ⟶ Inactive repressor (aporepressor)

→ No blocking of operator gene

→ *m*RNA transcription by structural genes.

→ Enzyme synthesis.

2. A corepressor present : Regulator gene ⟶ Inactive repressor (aporepressor)

→ + a corepressor ⟶ Active repressor

→ Binds to and blocks operator gene

→ No *m*RNA transcription by structural genes.

→ No Enzyme synthesis.

Negative Control : It has been seen that both in the inducible and repressible system, proteins syntehsis takes place when the operator gene is free and stops when it is blocked. Gene expression therefore only takes place when the operator gene is free. Such a control mechanism for protein synthesis is thus of the *negative type.* An example of negative control is the induction of protein synthesis in the presence of lactose and absence of glucose by the *lac* operon of *E. coli.*

In negative control the regular protein is the *repressor* and *it prevents gene transcription.* The controlling site on the operon is the *operator gene.* In the repressor function and thus enables gene transcription and protein synthesis. In the inducible system, the effector molecule is the *inducer.* The inducer provents *repressible system* the effector molecule is the *corepressor.* The corepressor stimulates repression function and thus prevents gene transcription.

Positive Control : In positive control the regulator protein acts as an *activator* and increases enzyme synthesis. The activator attaches to a separate controlling site, the *initiator site,* on DNA. The initiator site is not necessarily the operator gene. Since the regulator protein enhances gene transcription, this type of regulation is called *positive control* of protein synthesis.

The *ara* operon of *E. coli* (Fig. 23.20), which functions in the *utilization* of *arabinose,* is an example of positive control. The first three genes of the *ara* operon are *ara* D, *ara* A and *ara* B. These genes specify the synthesis of and *epimerase,* an *isomerase* and a *kinase,* respectively. An associated gene, ara C, functions as a *regulator gene.* The *ara* C gene may act as both *activator* and *repressor.* The *regulator protein* produced by the *ara* C gene may combine with *arabinose* (*inductr*) to form an *activator* protein. This activator protein binds to the *initiator site* (*ara* I) on te operon and enhances enzyme synthesis. In the absence of arabinose the regulator protein of *ara* C binds to the *operator* site (*ara* O), and thus acts as a *repressor.*

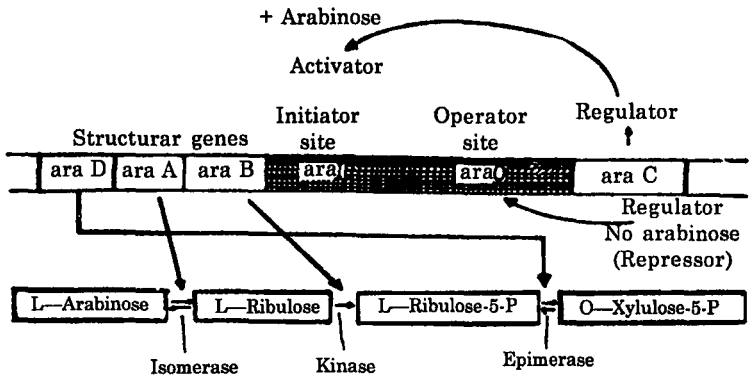


Fig. 23.20. The L-arabinose operon of *E. coli.*

Replication of DNA is an important example of positive control. Bacterial chromosomes consists of replicating units called *replicons* which are capable of independent genetic replication. One of the structural genes of the replicon produces an *activator* which is influenced by cytoplasmic stimuli. The activator interacts with the *replicator,* a particular section of the replicon, and initiates replication at this point.

Positive control may also be of the *inducible* or *repressible* type. In the *inducible system,* the effector molecule is the *inducer.* The inducer stimulates activator function and enables gene transcription and protein synthesis. In the *repressible system* the effector molecule is the *a corepressor.* The corepressor prevents activator function, and thus gene transcription.

Regulation of Gene Activity in Eukaryotes

Genes are active only when their products are required by the cell. The rest of the time they are 'switched off.' Thus in mouse liver cells only about 3% of the genes are active (*i.e.,* transcribe RNA), and in brain cells about 9% are turned on.

In *prokaryotes* control of gene activity takes place by means of special *repressor proteins* transcribed by *regulator genes.* Binding of the repressor protein to the *operator gene* blocks the movement of the transcribing enzyme RNA-*polymerase,* and thus prevents RNA transcription by the *structural genes.*

In *eukaryotes* gene regulation takes place by a different mechanism. The possible candidates for regulation of gene activity are the protein associated with DNA in the chromosomes. These include *histones* and *non-histone chromosomal proteins* (NHC proteins). It is unlikely that histones selectively repress gene activity since they have almost identical amino acid compositions in different organisms. It has been suggested that histones probably mask DNA in a *nonspecific* manner (*non-specific repression*).

Because histones are rich in *lysine* and *arginine* they have a *net positive charge* and are basic proteins. The positively charged groups, which appear to be arranged in clusters, could interact with the *negative charge* of the *phosphate backbone* of DNA. This would greatly reduce the charge repulsion of the phosphate groups and would facilitate *supercoiling* of DNA. The RNA *polymerase* molecule would not be able to move along the supercoiled DNA, and thus no transcription would be possible. It is thus possible that histones prevent transcription by bringing about supercoiling of DNA. This is shown by the fact that incorporation of RNA precursors in eukaryote cells take places only in regions of the chromosome where the chromatin is diffuse and not in the highly compact regions. The highly compact heterochromatic regions of the chromosome are therefore inert and cannot carry out transcription.

Among the models proposed to explain gene regulation in higher cells are (*i*) Frenster's (1965) model of gene-specific *derepressor* RNA, (*ii*) models in which *non-histone proteins* act as derepressors, *e.g.*, those proposed by Paul and co-workers (1971) and Stein *et al.* (1975) and (*iii*) Britten and Davidson's *operon-aperator model* (1969).

I. Frenster's model (Fig. 23.21). Histones stabilize the DNA double helix by interacting with the negative phosphate groups of DNA. This prevents the separation of strands, and consequently transcription. This prevents the separation of strands, and consequently transcription. The histones thus act as general *repressors* of protein synthesis. Histones are displaced from DNA by nuclear polyions which form complexes with histones. This permits separation of the DNA strands in a random manner. Gene-specific *derepressor* RNA hybridizes with the non-transcribing DNA strand at a particular locus and stabilises the loop in the open position. This frees the other DNA strand and permits it to transcribe messenger RNA.

II. Nonhistone proteins as derepressors : Paul and co-workers (1971) have suggested a similar model, except that in their model gene-specific *acid proteins* displace the histone. The *nonhistone chromosomal proteins* have a number of characteristics expected of a regulatory protein with specific action.

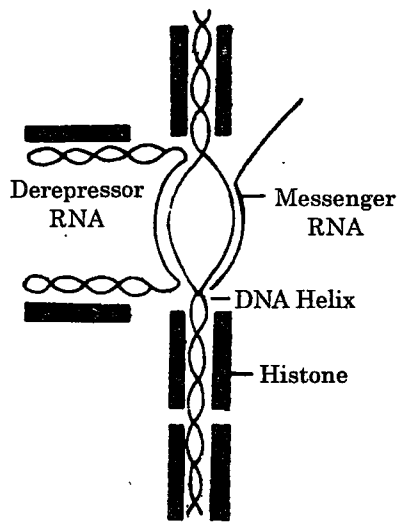


Fig. 23.21. Frenster's model of gene regulation.

1. NHC proteins occur in a far greater variety of forms than histones. They differ greatly in size and the length of the time they are associated with DNA before degradation.
2. The pattern of NHC proteins varies from species to species. Even different tissues in the same organism differ in their NHC protein pattern.
3. The pattern of NHC proteins varies with the developmental stage and also with the stage of the cell cycle. Thus NHC proteins isolated from interphase cells, where transcription of RNA is maximal, differ from those isolated from metaphasic cells, where only a few genes transcribe RNA.
4. NHC proteins have been localized in the active regions of the chromosomes.
5. Inhibition of transcription caused by histones is reversed on addition of NHC proteins, *i.e.*, NHC proteins act as *derepressors.*
6. Moreover, this derepression has *specificity.* NHC proteins *bind to specific gene loci.* This has been demonstrated as follows. Chromatin isolated from rabbit thymus and bone marrow was dissociated into DNA, histones and NHC protein. Reassociation of DNA and histones resulted in chromatin free from NHC protein. No transcription of RNA was observed in such preparations. RNA synthesis is completely suppressed by the histones. However, addition of NHC proteins from bone marrow resulted in transcription of RNA with characteritics of that of bone marrow. Similarly, addition of thymus NHC proteins resulted in RNA with characteritics of that found in thymus cells. This shows that NHC proteins are specific for a cell type. It is presumed that NHC proteins derepress specific genes and stimulate transcription of specific genetic regions.

   In another test this specificity is even more clearly demonstrated. Foetal mouse liver cells synthesisze the protein *globin* (the non-iron part of *haemoglobin*) but foetal mouse

brain cells do not. *In vitro* addition of NHC proteins from foetal liver to DNA and histone from the brain leads to production of globin mRNA.

7. NHC proteins show preferential binding to the DNA of the organisms from which they are isolated.

According to the theory of Stein and his coworkers (see Stein, G.S., Stein J.S. and Kleinsmith, 1975), regulations gene activity takes place as follows (Fig. 23.22). Histones probably act as *nonspecific repressors* of genetic function and prevent transcription of RNA from DNA. Individual NHC protein recognize certain sites on DNA and bind to it there. They then pull off the histone repressor from the site, thus *derepressing* DNA. NHC proteins act by derepressing.

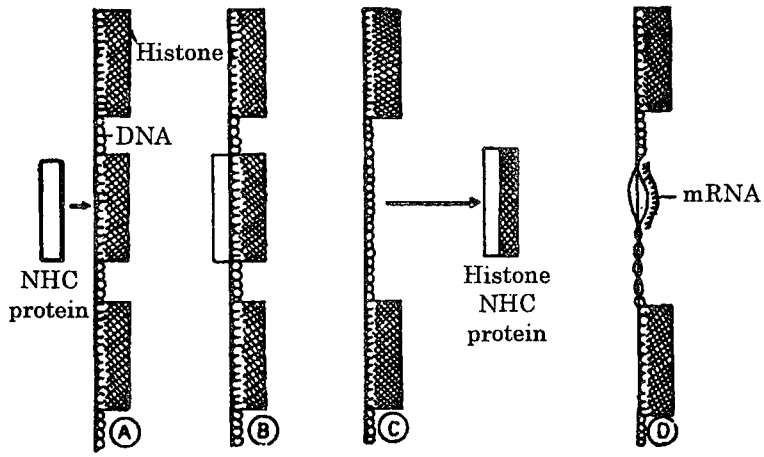


Fig. 23.22. Model of gene regulation by removal of histones.

A. Non-specific making of DNA by histones represses DNA.
B. Non-histone chromosomal (NHC) protein binds to specific sites on DNA.
C. NHC protein pulls out histone repressor.
D. Unmasked DNA is derepressed and transcribes mRNA.

III. The Britten-Davidson (1969) operon-operator model (Fig. 23.23) for regulation in higher cells includes certain features which are not found in the simpler Jacob Monod model for bacterial cells. According to this speculative model four basic types of genes are visualized, *sensor genes, producer genes integrator genes* and *receptor genes.*

1. *Sensor genes* are detectors which are sensitive to the state of the cell and its environment. The sensor gene is stimulated by various substances like enzymes, hormones and metabolites, perhaps through intermediary molecules.

2. *Producer genes* are the output controls of the regulation mechnism. They specify control for the formation of cell structures like membranes, organelles, etc., and operator substances like enzymes, etc.

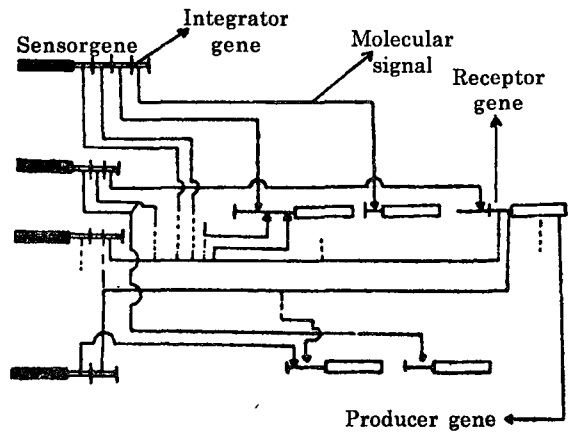


Fig. 23.23. The Britten-Davidson generalized 'operon-operator' models for gene regulation in eukaryote cells.

3. *An integrator gene* may be associated with a gensor gene, or several integrators may be associated with a sensor. The integrator gene sends out a specific signal (molecule) to other genes when the sensor gene is activated. When several integrators are associated with a single sensor, the sensor can initiate a variety of signals.

4. *A receptor gene* is associated with a single producer gene, and is a link between the integrator gene and the producer gene. When the recreptor gene receives an integrator signals the producer gene is activated. A particular producer gene may have many receptors associated with it.

Role of Interferons

Introduction : Hoskins (1953) found that monkeys injected with one strain of the yellow fever virus were protected against a second strain. Similarly Findlay and MacCallum (1935) showed that the Rift Valley fever virus protected monkeys from the yellow fever virus. They called this protective effect *virus interference*. In 1957 Issacs and Lindenmann discovered that viral interference was brought about by a substance, which they called *interferon,* produced by the injected cell.

Cells injected by viruses produce interferon, which is anti-viral in action. it spreads to neighbouring cells and makes them resistant to virus infection by inhibiting virus growth (Fig. 23.24). A virus entering a cell containing interferon cannot multiply.

Interferons act against a wide variety of viruses, *i.e.,* they are not virus specific. They are, however, *species specific.* Interferon from one organism does not give protection against viruses to cells of another organism.

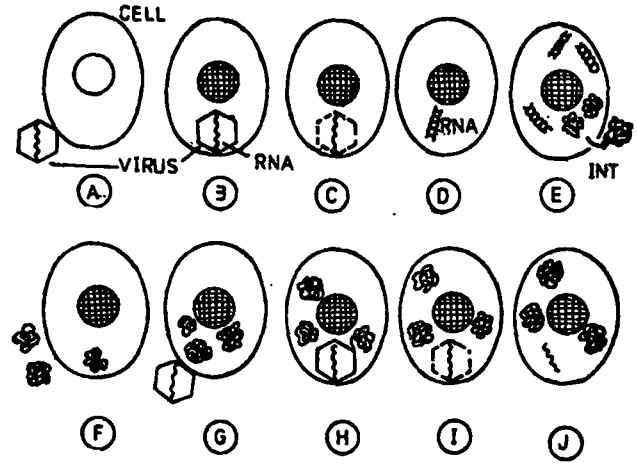


Fig. 23.24. Formation and action of interferon.
Nu—nucleus. INT—interferon.
A. Virus attaches to cell.
B. Virus enters cell.
C. Virus coat removed releasing RNA.
D. RNA replicates to form double stand.
E. Presence of RNA induces cell to synthesize interferon.
F. Interferon enters new cell.
G. Virus attaches to cell.
H. Virus enters new cell.
I. Virus coat removed releasing RNA.
J. RNA, however, cannot replicate in the presence of interferon.

Nature of Interferons : Interferons are proteinaceous substances which are produced inside the body as a defense against virus infection. In the laboratory tests, double stranded synthetic RNA has also been able to induce the synthesis of interferons in the mammalian cells. It has been possible to purify many interferons and their amino acid composition has been ascertained.

Difference between Interferon and Antibodies : Although both are proteins, interferon differs from antibodies in many ways. Anti-bodies are formed in specialized cells, while interferon is formed only in infected cells. Antibodies are specific against a particular virus or a closely related groups of viruses, while interferon acts against many different viruses. Antibodies combine with virus particles, while interferon acts by interfering with protein synthesis.

Action of Interferons : It has been reported that interferons control virus infection in the following two ways :

(*i*) The interferons affect the cellular membranes, so that the virus particles do not get disseminated to the other cells.

(*ii*) Interferon affects viral synthesis by interfering with the binding of viral *m*RNA with ribosomes. Thus protein synthesis is inhibited, and new viruses cannot be formed. It is belived that interferon acts by inducing a cellular gene to produce an inhibitor, which then interferes with ribosome function. A single interferon molecule is sufficient to induce inhibitor formation. Thus, interferon acts in extremely small amounts.

It has been reported that ribosomes from viral interferon infected mammalian cells fail to form polysomes with viral RNA, but if they are exposed to host (mammalian) RNA they readily form polysomes and protein synthesis gets initiated. This reveals that ribosomes can recognise that specific m-RNA and could regulate the overall protein synthesis.

Since interferon acts against a wide variety of viruses it has the potential as an effective anti-viral agent. However, no practical general method has been found to produce interferon in human cells. Vaccination therefore continues to be the only practical way of controlling virus-borne diseases in man.

24 Redox Potential, Electron Transport and Oxidative Phosphorylation

24.1 Introduction

The various enzyme-catalyzed steps in the oxidative degradation of carbohydrates, fats and amino acids in aerobic cells get converged into electron transport and oxidative phosphorylation, the final stage of cell respiration. This stage involves the flow of electrons from organic substrates to oxygen with the simultaneous release of energy for the generation of ATP molecules. The significance of this final stage of respiration in the human body can be realized by the fact that a normal adult businessman having a 70 kg weight needs about 2,800 kcal of energy per day. The amount of energy could be produced by the hydrolysis of about 2,800/7.3 = 380 mole or 190 kilograms of ATP. However, the total amount of ATP present in his body is somewhat 50 grams. In order to provide chemical energy for the body need, the 50 g of ATP must be broken down into ADP and phosphate and resynthesized thousand of times in a day, *i.e.*, 24 hours.

24.2 Electron Flow as a Source of ATP Energy

During each turn of citric acid, there occurs the elimination of 4 pairs of hydrogen atoms one each from isocitrate, α-ketoglutarate, succinate and malate, by the action of specific dehydrogenases. These hydrogen atoms on donating their electrons to the electron transport chain become H^+ ions, which enter into the aqueous medium. These electrons get transported along a chain of electron carrying molecules to ultimately reach cytochrome aa_3 or cytochrome oxidase, which promotes the transfer of electrons to oxygen, the final electron acceptor in aerobic organisms. As each atom of oxygen is accepting two electrons from the chain, two H^+ are taken up from the aqueous medium to form water.

Besides the citric acid cycle, there are other pairs of hydrogen atoms which are also released from the dehydrogenases that act upon pyruvate, fatty acids and amino acids during their degradation to acetyl-CoA and other products. All these hydrogen atoms virtually donate their electrons ultimately to the respiratory chain with oxygen as the terminal electron acceptor.

The respiratory chain possesses a series of proteins having tightly bound prosthetic groups which are capable of accepting and donating electrons. Each member of the chain is able to accept electrons from the preceding member and transfers them to the following one, in a specific sequence. The electrons entering the electron-transport chain have been energy-rich, but as they pass down the chain step-by step to oxygen, they lose free energy. Much of this energy gets conserved in the form of ATP in the inner mitochondrial membrane. As each pair of electrons passes down the respiratory chain from NADH to oxygen, the synthesis of 3 moles of ATP from ADP and phosphate occurs. The 3 segments of the respiratory chain that provide energy to generate ATP by oxidative phosphorylation are termed as the *energy conserving sites.*

24.3 Electron-Transferring Reactions

Chemical processes in which electrons get transferred from one molecule to another are termed as *oxidation-reduction reactions* or *oxidoreductions* or *redox reactions.* In fact, the electron-transferring reactions have been oxidation reduction reactions. The electron-donating molecule in such a reaction is termed as the *reducing agent* (= *reductant*) and the electron-accepting molecule as the *oxidzing agent* (=*oxidant*). The reducing or oxidizing agents function as *conjugate reductant-oxidant pairs* (*redox pairs*). The general equation may be put as follows :

$$\text{Electron donor} \rightleftharpoons e^- + \text{Electron acceptor}$$

A specific example has been the reaction,

$$Fe^{2+} \rightleftharpoons e^- + Fe^{3+}$$

where ferrous ion (Fe^{2+}) has been the electron donor and the ferric ion (Fe^{3+}) the electron acceptor. Fe^{2+} and Fe^{3+} together is termed as a conjugate redox pair.

Electrons get transferred from one molecule to another according to one of the following ways (Lehninger, 1984).

1. Directly in the form of electrons : For example, the $Fe^{2+} - Fe^{3+}$ redox pair is able to transfer an electron to the $Cu^+ - Cu^{2+}$ pair.

$$Fe^{2+} + Cu^{2+} \longrightarrow Fe^{3+} + Cu^+$$

2. In the form of hydrogen atoms : A hydrogen atom has a proton (H^+) and a single electron (e^-). The general equation may be put as follows :

$$AH_2 \rightleftharpoons A + 2e^- + 2H^+$$

in which AH_2 has been the hydrogen (or electron) donor. A is the hydrogen acceptor and AH_2 and A together constitute a conjugate redox pair. This redox pair is able to reduce the electron acceptor B by the transfer of H atoms as follows :

$$AH_2 + B \longrightarrow A + BH_2$$

3. In the form of a hyride ion : The hyride ion (: H^-) is having two electrons, as in the case of NAD linked dehydrogenases.

4. *During direct combination of an organic reductant with oxygen :* In these reactions a product gets formed in which the oxygen is covalently incorporated, for example in the oxidation of a hydrocarbon to an alcohol,

$$R{-}CH_3 + \frac{1}{2}\,O_2 \longrightarrow R{-}CH_2{-}OH$$

where the hydrocarbon has been the electron donor and the oxygen atom, the electron acceptor.

All four types of electron transfer take place in cells. The neutral term *reducing equivalent* is generally used to refer to a single electron equivalent participating in redox reactions, whether it has been in the form of an electron *perise,* a hydrogen atom, a hydride ion or as a reaction with oxygen to yield an oxygenated to lose two reducing equivalents at a time and also as each oxygen atom can accept two reducing equivalents, it has been customary to treat the unit of biological oxidations as a pair a reducing equivalents passing from substrate to oxygen.

24.4 Standard Redox Potential or Standard Oxidation Reduction Potential

The tendency of any particular atom, ion or molecule to lose an electron, thereby being oxidized, is measured by its standard oxidation reduction potential, *i.e,* which is also known as redox potential.

Redox potential is the potential of a platinum electrode placed in a solution of equimolar concentrations of both the reduced and oxdised forms of the substance and measured against the standard hydrogen electrode. The more strongly reducing the substance, the more negative is E_0. Thus substance A with a more positive E_0 is able to oxidise substance with a more negative E_0. Thus, the quantitative measure of the affinity of a compound to lose or gain electrons is the redox potential (reduction-oxidation potential).

Standard redox potential is denoted by the symbol E_0' and is defined as the electromotive force (e.m.f.) in volts given by a responsive electrode placed in solution having both the electron donor and its conjugate electron acceptor at 1.0 M concentration, 25°C and 7.0 pH. Although standard potentials are expressed in units of *volts,* they are often expressed in *millivolts* for convenience. Each conjugate redox pair possesses a characteristic standard redox potential. By convention, the standard potentials of conjugate redox pairs are expressed as reduction potentials, which assign increasingly negative values to systems having an increasing tendency to lose electrons, and increasingly positive values to systems having an increasing tendency to accept electrons. In other words, the more negative the E_0' the lower is the affinity of the system for electrons and conversely, the more positive the E_0' of a system, the greater has been its electron affinity. Hence, electrons tend to flow from one redox couple to another in the direction of the more positive system.

Measurement of Redox Potential of an Organic Compound

Redox potential of an organic compound can be determined by using a standard platinum electrode as shown in Fig. 24.1. One electrode is made of platinum which being an inert material does not dissolve in solution to form ion. One compartment of the apparatus has reducing system in buffer. Nitrogen gas is made to pass through the system continuously. The known amount of oxidant is added and the mixture has to be stirred continuously. Potential of the mixture is measured by using potentiometer through platinum and hydrogen or calomel electrodes. Hydrogen electrode is dipped in a different compartment having KCl solution and connected to the first compartment through a salt bridge which contains KCl-gelatin solution.

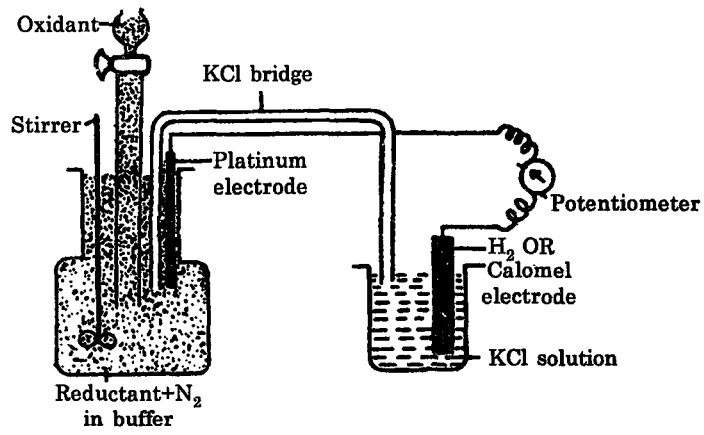


Fig. 24.1. Arrangement to measure redox potential of an organic compound.

Redox potential can be known indirectly from Peter's equation which is as follows :

$$\text{Redox potential (E)} = E_o + \frac{RT}{nF} \ln \frac{[\text{oxidant}]}{[\text{reductant}]}$$

where E_o represents the redox potential of mixture having equimolal concentrations of the oxidant and the reductant, R gas constant in joules per mole degree, T absolute temperature, F Faraday number, *n* the number of electrons per g equivalent of reactant and the logarithm to the base *e*. It is possible to simplify Peter's a equation under normal conditions of temperature 30°C (absolute 303°), valency change of 2 and converting into $\log_{10}$ may be put in the following way :

$$E = E_o + 2.303 \log_{10} \frac{[\text{oxidant}]}{[\text{reductant}]}$$

If the concentration of oxidant becomes equal to the concentration of reductant, the factor, $\log_{10} \frac{[\text{oxidant}]}{[\text{reductant}]}$ will become zero and $E = E_o$. The E_o values for some of the biological redox systems have been given in Table 24.1

Table 24.1

STANDARD REDOX POTENTIALS, E_o' OF SOME REDOX PAIRS PARTICIPATING IN OXIDATIVE METABOLISM

Redox couple	E_o' *(in volts)*
Some substrate couples	
Acetyl-CoA + CO_2 + $2H^+$ + $2e^-$ ⟶ Pyruvate + CoA	– 0.48
α-ketoglutarate + CO_2 + $2H^+$ + $2e^-$ ⟶ isocitrate	– 0.38
3-phosphoglyceroyl phosphate + $2H^+$ + $2e^-$ ⟶ Glyceraldehyde-3-phosphate + Pi	– 0.29
Pyruvate + $2H^+$ + $2e^-$ ⟶ Lactate	– 0.19
Oxaloacetate + $2H^+$ + $2e^-$ ⟶ Malate	– 0.18
Fumarate + $2H^+$ + $2e^-$ ⟶ Succinate	+ 0.03
Components of the electron-transport chain	
$2H^+$ + $2e^-$ ⟶ H_2	– 0.41
NAD^+ + H^+ + $2e^-$ ⟶ NADH	– 0.32
$NADP^+$ + H^+ + $2e^-$ ⟶ NADPH	– 0.32
NADH dehydrogenase (FMN form) + $2H^+$ + $2e^-$ ⟶ NADH dehydrogenase ($FMNH_2$ form)	– 0.30
Ubiquinone + $2H^+$ + $2e^-$ ⟶ Ubiquinol	+ 0.04
Cytochrome *b* (oxi) + e^- ⟶ Cytochrome *b* (red.)	+ 0.07
Cytochrome c_1 (oxi.) + e^- ⟶ Cytochrome c_1 (red.)	+ 0.23
Cytochrome c_1 (oxi.) + e^- ⟶ Cytochrome *c* (red.)	+ 0.25
Cytochrome *a* (oxi) + e^- ⟶ Cytochrome *a* (red.)	+ 0.29
Cytochrome a_3 (oxi.) e^- ⟶ Cytochrome a_3 (red.)	+ 0.55
$^{1/2}O_2$ + $2H^+$ + $2e^-$ ⟶ H_2O	**+ 0.82**

* Assuming 1 M concentrations of all components, pH = 7.0 and temperature = 25°C.

E_0' refers to the partial reaction written as :

$$\text{Oxidant} + e^- \longrightarrow \text{Reductant}$$

Note the two landmark potentials which are for the $H_2/2H^+$ and the $H_2O/^{1/2}O_2$ couple.

In Table 24.1 the standard redox potentials of some systems have been included which are useful in biological electron transport. They have been listed in order of increasing potential, *i.e.*, in the order of decreasing tendency to lose electrons. Thus, conjugate redox pairs having relatively negative standard potential tend to lose electrons to those lower in the table. For example, when the isocitrate/α-ketoglutarate + CO_2 couple is present in 1.0 concentration, it has a standard potential E_0' of –0.3.8 V. This redox couple tends to pass electrons to the redox couple NADH/NAD^+, which has a relatively more positive potential, in the presence of isocitrate dehydrogenase. Conversely, the strongly positive standard potential of H_2O/O_2 couple, 0.82 V, reveals that water molecule has very little tendency to lose electrons to form molecular oxygen. In other words, molecular oxygen has a very high affinity for electrons or hydrogen atoms.

In oxidation systems, the electrons will tend to flow from a relatively electronegative conjugate redox pair, such as NADH/NAD^+ ($E_0' = -0.32$ V), to the more electropositive pair, such as reduced cytochrome c/oxidized cytochrome c($E_0 = + 0.23$ V). Likewise, they will also tend to flow from the cytochrome c redox pair to the water/oxygen pair ($E_0' = + 0.82$ V). The greater the difference in the standard potentials between two redox pairs, the greater is the free energy loss as electrons pass from the electronegative to the electro positive pair. Therefore, when electrons flow down the complete electron-transport chain from NADH to oxygen *via* several electron-carrying molecules, they lose a large amount of free energy.

Effect of pH on Redox Potential : In many oxidation-reduction reactions not only electron transfer but also hydrogen transfer takes place. For example, the oxidation of hydroquinone to quinone liberates H^+ also.

$$\underset{\text{Hydroquinone}}{H_2Q} \underset{\text{reduction}}{\overset{\text{oxidation}}{\rightleftharpoons}} \underset{\text{Quinone}}{Q} + 2H^+ + 2e$$

In these reactions, hydrogen ion concentration (pH) is expected to effect the oxidation-reduction process in accordance to the law of mass action. In the redox system of Fe^{3+}/Fe^{2+} however, where hydrogen transfer is not involved, pH does not have any effect on the system.

In order to calculate the redox potential of hydrogen involving systems, Peter's equation may be put in the following form :

$$E = E_0' + \frac{RT}{nF} \ln \frac{[\text{oxidant}]}{[\text{reductant}]} + \frac{RT}{F} \ln [H^+]$$

If the latter half of equation is not considered, it must be stated that at what pH, E_0 is being measured. In that case E_0 will be written as E_0' which is symbolising standard redox potential at a known pH.

Redox System and Free Energy : The difference in redox potential, ΔE_0 between an oxidising and a reducing agent is related to the free energy change which takes place when the oxidising agent is reduced by the reducing agent by the following expression :

$$\Delta G^{0\prime} = -nF\Delta E_0'$$

where n represents the number of electrons transferred and F is Faraday's constant (66230 g/V equivalent). For example, the oxidation of NADH by molecular oxygen can be represented by the following equation.

$$H^+ + \text{NADH} + \frac{1}{2} O_2 \longrightarrow NAD^+ + H_2O$$

In the above reaction, $n = 2$ and E_0 is found out by taking the difference between E_0 for

$NADH/NAD^+$ (–0.320 V) and for water/oxygen (0.816 V) both at pH 7.0, *i.e.*,

$\Delta E_0 = 0.816 - (-0.320) = 1.136V$. Hence, on substituting the various values in Eq. (1), we get

$\Delta G^{0'} = (-2)\,(96530)\,(1.136) = -218.6$ kJ/mol

As $\Delta G^{0'}$ for this reaction has been found to be large and negative, it implies that the oxidation of NADH is strongly exergonic. This favourable free energy change is not indicative of the fact that NADH gets rapidly oxidised by molecular oxygen in the absence of a catalyst. However, up to three molecules of ATP were formed from ADP and phosphate at the expense of oxidation of NADH to NAD^+ with molecular oxygen. Thus, the overall equation for the respiratory chain phosphorylation could then be written as

$$NADH + H^+ + 3\,ADP + 3\,Pi + \frac{1}{2}O_2 \longrightarrow NAD^+ + 4H_2O + 3\,ATP$$

This reaction equation can be separated into an exergonic component

$$NADH + H^+ + \frac{1}{2}O_2 \longrightarrow NAD^+ + H_2O;$$

$$\Delta G^{0'} = -221.34 \text{ kJ mol}^{-1}$$

and an endergonic component,

$$3ADP + 3Pi \longrightarrow 3ATP + 3H_2O$$

$$\Delta G^{0'} = 91.98 \text{ kJ mol}^{-1}$$

Coupled phosphorylation of three molecules, of ATP thus conserves $91.98/221.34 \times 100$ or about 42 per cent of the total free energy decrease during transport of a pair of electrons from NADH to oxygen under the usual standard conditions.

By doing the calculations of the free energy change using $\Delta G_0' = -n\mathrm{F}\Delta E_0'$, it becomes possible to predict the approximate sites of the three energy-delivering segments of the respiratory chain. In Fig. 24.2, three spans in the chain have been shown. In each span, relative large decrease in free energy takes place which may be sufficient to provide the energy for the formation of ATP from ADP and phosphate. Experimentally, it was proved that three spans are actually the energy-coupling sites of the respiratory chain. These are designated as *site I* (the span between NADH and coenzyme Q), *site II* (the span between cytochrome *b* and cytochrome *c*) and *site III* (between cytochrome *a* and oxygen). Three of these spans provide sufficient energy to produce a molecule of ATP from ADP and phosphate. Thus, the respiratory chain may be regarded as the energy transforming device.

The precise sequence and function of all the oxidation-reduction centres is not exactly known.

Fig. 24.3 is an energy diagram showing (*a*) the standard potentials of the electron carriers of the respiratory chain, (*b*) the direction of electron flow, which is always "downhill" toward oxygen and (*c*) the relative free-energy change at each step.

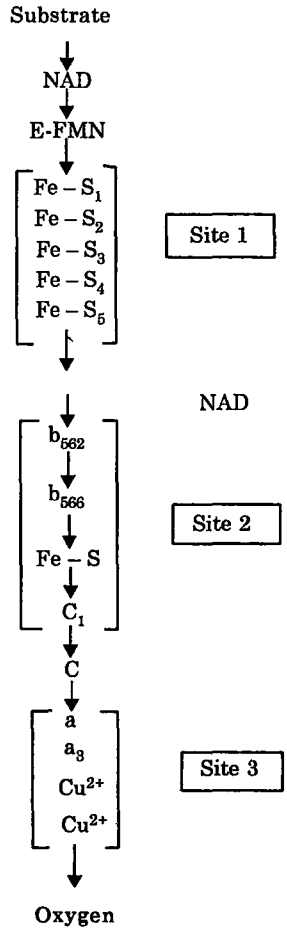


Fig. 24.2. The complete set of electron carriers of the respiratory chain.

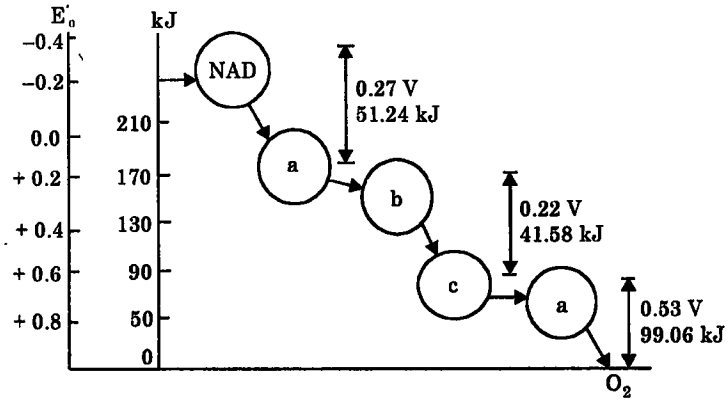


Fig. 24.3. Redox potential and generation of ATP in the electron transport system.

Electrons are transferred from substrate to oxygen though a series of electron carriers-flavins, iron-sulphur complexes, quinones and hemes.

Site of Oxidative Phosphorylation

Mitochondria is the site of carrying out oxidative phosphorylation.

Mitochondria are small granular or filamentous bodies which are called the powerhouses of the cell. They are associated with cellular respiration and are the sources of energy.

Size : The average length of the mitochondrion is 3-4 microns and the average diameter 0.5 to 1.0 microns. In muscles, most of the mitochondria are 2-3 microns long, but bodies 8-10 microns long have also been found.

Shape : Mitochondria vary in shape but are generally granular of filamentous. If a filamentous mitochondrion swells at one end, it gives a club-shaped appearance. If the swollen end hollows out then the appearance is that of a tennis racket. Pleomorphic forms may contain swellings at both ends. If there is a central clear zone the mitochondrion becomes vesicular.

Number : The number of mitochondria varies in different cell types. It is however, rather constant for a particular cell type. Mitochondria are present in all cells which respire aerobically. They are absent in bacteria, where the plasma membrane has respiratory enzymes. The number of mitochondria depends upon the metabolic activity of the cell. Cells with high metabolic activity have a high number of mitochondria, while those with low metabolic activity have a lower number. In a normal liver cell there are 1000–1600 mitochondria. Large sea urchin eggs have 13,000–14,000, while renal tubules have 300–400. In the sperm there are as few as 20–24 mitochondria while in some oocytes there are about 300,000.

Distribution : Ordinarily mitochondria are evently distributed in the cytoplasm. They may, however, be localized in certain regions. In the proximal convoluted tubules of the kidney they are found in the *basal* region of the cell, opposite the renal capillaries. In skeletal muscles they lie between the myofibrils. In insect flight muscle several large mitochondria are in contact with each fibril. (Fig. 24.4 A). In cardiac muscle the mitochondria are situated in clefts between the myofibrils. Numerous lipid droplets are associated with the mitochondria (Fig. 24.4B). In many sperms the mitochondria fuse into one or two structures which lie in the middle piece of the tail, surrounding the axial filament (Fig. 24.4 C). In columnar or prismatic cells they are oriented parallel to the long axis of the cell. In leucocytes they are radially arranged.

Movement : Mitochondria may move freely in some cells, carrying ATP wherever required, or may be localized in a particular region, as in muscles and in reptilian venom glands. Movement is less in animals than in plants. Phase contrast studies on fibroblasts have shown that mitochondria change shape and volume. Often the movements are rhythmical.

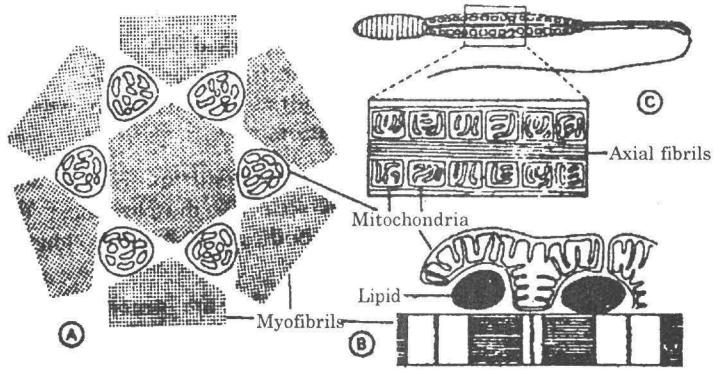


Fig. 24.4. A. Arrangement of mitochondria in insect (*caliphora*) flight muscle.
B. Mitochondria in pipillary muscle of cat heart.
C. Mitochondria arranged around axial filament in mammalian sperm.

Structure of Mitochondria

The mitochondrion is bounded by two membranes, the *outer membrane* and the *inner membrane* (Fig. 24.5). The space between the two membranes is called the *outer chamber of inter-membrane* space. It is filled with a watery fluid, and is 40-70A in width. The space bounded by the inner chamber is called *inner chamber* or *inner membrane space.* The inner membrane space is filled with a *matrix* which contains dense *granules* (300-500A), ribosomes and *mitochondrial* DNA. The *granules* consist of insoluble inorganic salts and are believed to be the binding sites of divalent ion like Mg^{2+} and Ca^{2+}. In some cases they apparently contain polymers of sugars. The side of the inner membrane facing the matrix side is called the M-side, while the side facing the outer chamber is called the C-*side.* Two to six *circular* DNA *molecules* have been identified within mitochondria. These rings may either be in the open or in the twisted configuration. They may be present free in the matrix or may be attached to the membrane. The *enzymes* of the Krebs cycle are located in the matrix.

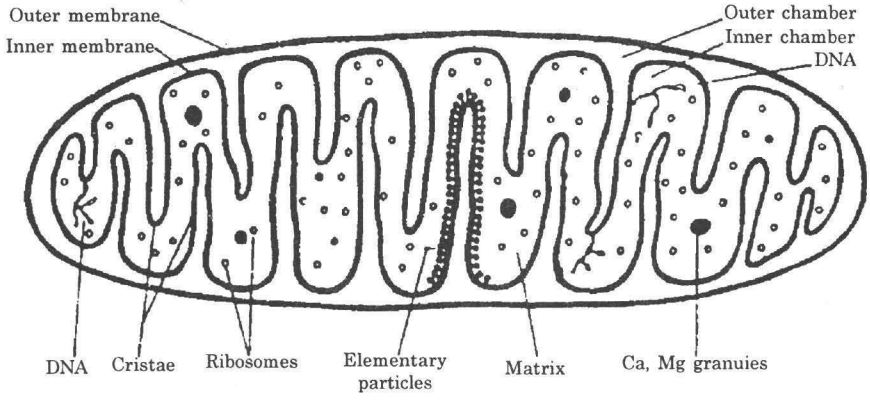


Fig. 24.5. Section through mitochondrion (diagrammatic).
The elementary particles have been shown on only one crista.

The inner membrane is thrown up into a series of folds, called *cristae mitochondriales,* which project into the inner chamber. The cavity of the cristae is called the *intercristae space,* and is continuous with the intermembrane space.

According to earlier descriptions (Green and Perdue, 1966) the outer surface of the outer membrane and the inner surface of the inner membrane were supposed to be covered with thousands of small particles. Those on the outer membrane were described as being stalkless and were called the *subunits of Parson.* The stalked inner membrane particles were called the *subunits of Fernandez-Moran.* In recent works only the stalked particles of the inner membrane are considered to be involved in hydrogen transport.

Mitochondrial Membranes

Both the outer and the inner mitochondrial membranes are about 60A-70A thick. The intermembrane space separating them is 40-70A. Both structurally and functionally the two membranes are entirely different.

1. Cristae : The outer membrane is smooth while the inner membrane is thrown up into folds and tubules (the *cristae*).

2. Electron transfer and ATP synthesis : The electron transfer and ATP synthesizing systems are located only in the inner membrane. Since the matrix contains the enzymes of the Krebs cycle, the inner mitochondrial membrane and the matrix together constitute the respiratory unit.

3. Lipids (Fig. 22.6A) *:* The relative proportions of the phospholipids of guinea pig liver (Parsons et al., 1967) are given in the following table :

Tabel 24.1

Lipid	*Outer Membrane*	*Inner Membrane*
Phosphatidylinositol	13.5	4.2
Phosphatidylcholine	55.2	45.5
Phosphatidylethanolamine	25.3	27.7
Cardiolipin	3.2	21.5
Others	2.8	2.1

In general the outer membrane contains more phospholipid (× 3) and more cholesterol (× 6) than the inner membrane. Phosphatidylcholine is the predominant lipid of the outer membrane. The inner membrane on the other hand contains most of the diphosphatidylglycerol (cardiolipin) of the mitochondrion.

4. Proteins : The outer membrane contains less than 10% of the total protein of the mitochondrion while the inner membrane contains as much as 60%. The outer membrane contains about 14 different proteins with molecular weights ranging from 12,000 to 220,000. The inner

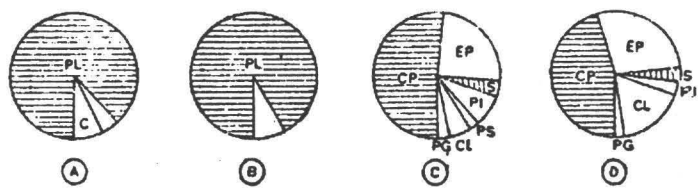


Fig. 24.6. A Lipid composition by weight of :
A : Outer mitochondrial membrane
B : Inner mitochondrial membrane
C : Outer mitochondrial membrane
D : Inner mitochondrial membrane
PL : Phospholipid
C : Cholesterol
CP : Choline phosphoglycerides
EP : Ethanolamine phosphoglycerides
S : Sphingomyelin
PS : Phosphatidylserine.

membrane contains a large number of proteins (associated with heme, flavin, copper and non-heme iron moieties) ranging in molecular weight from 10,000 to 90,000. Most of the proteins are associated into five complexes (complexes I-V). Complexes I-IV are electron-transfer complexes and Complex-V the ATP synthesizing complex.

5. Enzymes : The outer membrane has a variety of enzymes, but none closely associated with respiration or oxidative phsophorylation. As mentioned previously, the electron transfer and ATP synthesizing enzymes are located in the inner membrane.

Outer membrane enzymes	*Inner membrane enzymes*
Monoamine oxidase	ATPase
NADH-cytochrome reductase	Carnitine acetyltransferase
Glycerophosphate acyltransferase	Steroid 11-B-hydroxylase
Hexokinase 2	Succinate dehydrogenase
Cholinephosphotransferase	Choline dehydrogenase
Acyl-Co synthtease	Cytochrome oxidase
	3-Hydroxybutyrate dehydrogenase

6. Sialic acid : The outer membrane has × 4 to × 5 as much sialic acid as the inner membrane. The sialic acid is associated with glycolipid and glycoprotein.

7. Ion transport components : The inner membrane contains several ion-transport components for transporting ions such as phosphate, ADP, ATP, dicaroboxylate, tricarboxylate, glutarate, α-ketoglytarate, asparate, pyruvate, citrulline, ornithine, bicarbonate + CO_2, calcium, magnesium and sodium.

8. Diffusion : Substances upto MW 10,000 daltons can pass through the outer membrane by passive diffusion. On the other hand, except for uncharged molecules of MW, <100 daltons, most substances cannot pass through the inner membrane by simple diffusion.

Cristae : The cristae are infoldings of the inner membrane. The arrangement of the cristae varies in mitochondria from different sources (Fig. 24.7). Generally the cristae are in the form of *folds*. In the mitochondria of the adrenal cortex of the hamster, however, the cristae are *tubular*. These cristae appear as circles in transverse section. Tubular cristae are also found in mitochondria of brown adipose tissues of the hibernating bat, and extend all the way across the organelle. In hamster liver mitochondria there is a mixture of folded and tubular cristae.

In relatively inactive cells, *e.g.*, the live cells of the salamander, the internal structure of the mitochondria is simple, and there are few cristae. In the liver cells of the bat *Myotis* there are plate-like cristae of varying length. In the bat cricoid muscle there are several long cristae, closely packed together in a parallel arrangement.

In glandular cells from the gastric mucosa of the frog the cristae have sharp angulations at regular intervals. The angulations give a zigzag appearance to the cristae. Angular cristae are more often found in active tissues like skeletal or cardiac muscle. In the cat ventricular papillary muscle the angulations fuse in certain places, giving rise to cylindrical areas of matrix.

In the normal summer frog with high cytochrome oxidase activity the cristae of mitochondria from the proximal tubule are typically transverse. In the starved winter frog with low cytochrome oxidase activity the cristae show a longitudinal arrangement. Some mitochondria have both longitudinal and transverse cristae.

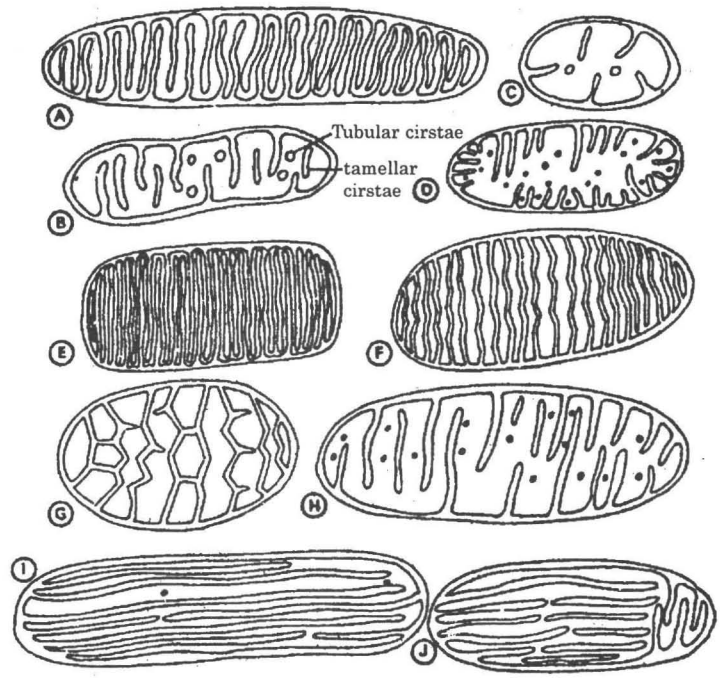


Fig. 24.7. Variation of cristae in mitochondria. (A) Mitochondrion from the adrenal cortex of the hamster with tubular cristae extending across the full width. (B) From hamster liver showing mixture of lamellar and tubular cristae. (C) From liver cell of salamander showing few cristae and few granules in the matrix. (D) From the liver cell of the bat *Myotis* showing relative short plate-like cristae of varying lengths and many granules. (E) From bat cricothyroid muscle showing many long, closely packed, parallel cristae and few granules. (F) From glandular cell of gastric mucosa of frog showing cristae with sharp angulations. (G) From cat ventricular papillary muscles showing fusion of cristae to produce a honey comb of cylindrical areas of matrix. (H) From proximal tubule of normal summer frog showing normal cristae and numerous granules. (I) From proximal tubule of starved winter from showing longitudinal cristae and few granules. (J) Transverse and longitudinal cristae.

Mitochondrial Particles

In earlier descriptions, particles were described on the outer membrane and were called the *subunits of Parson.* These were considered to be hollow cylinders, 60A long and 60A wide, with a central hole 20A in diameter. The centre to centre spacing between the subunits was described as 80A. The outer membrane particles were thought to contain the enzymes of the Krebs cycle. It has, however, been shown that the enzymes of the Krebs cycle are located in the matrix of the mitochondrion (Lehininger, 1969).

Associated with the inner membrane are several thousand small particles which have been called *elementary particles* or *subunits of Fernandez-Moran,* or F_0–F_1 complex or ATPase complex. Each particle consists of a *basepiece,* a stalk, a *headpiece.* The particles are spaced about 100A intervals. The headpiece is 75-100A in diameter, and the stalk about 50A in length.

It was formerly thought that each elementary particle contained all the enzymes for electron transport and oxidative phosphorylation. The particles were therefore termed *electron transfer particles (ETP).* Each particle was believed to consist of four complexes, with complexes I and II situated in the basepiece, complex III in the stalk and complex IV in the head piece (Green, 1064) (Fig. 24.8).

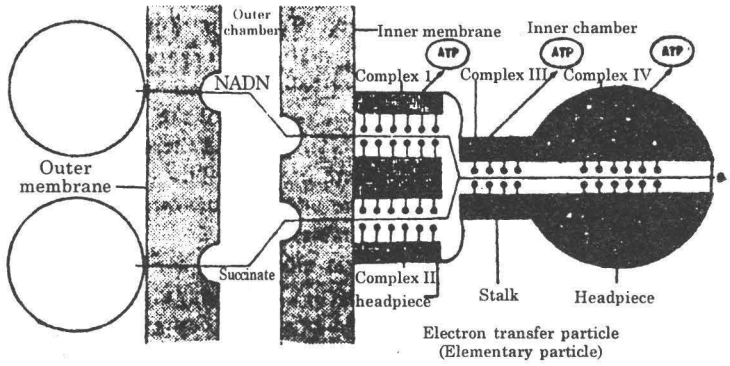


Fig. 24.8. The old concept of the arrangement of complexes. I-IV in the elementary particle (From Green, 1964).

In later studies it was suggested that the respiratory chain lies in the basepieces. Each basepiece was believed to correspond to a complex containing a part of the enzymes of the chain (Fig. 24.9).

According to current views the headpiece (F_1) contains *ATPase proper*, the stalk consists of F_5 or *oligomycin sensitivity conferring protein* (CSCP) and F_6 (Fc_2), and the basepiece (F_0) contains the *proton channel*. The enzymes and other components of the respiratory chain are embedded within the inner mitochondrial membrane (Fig. 24.10).

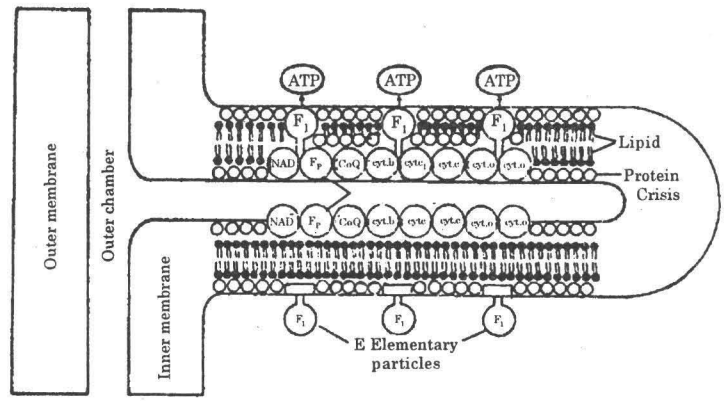


Fig. 24.9. A later version (also now obsolete) of the arrangement of the respiratory chain in the crista.

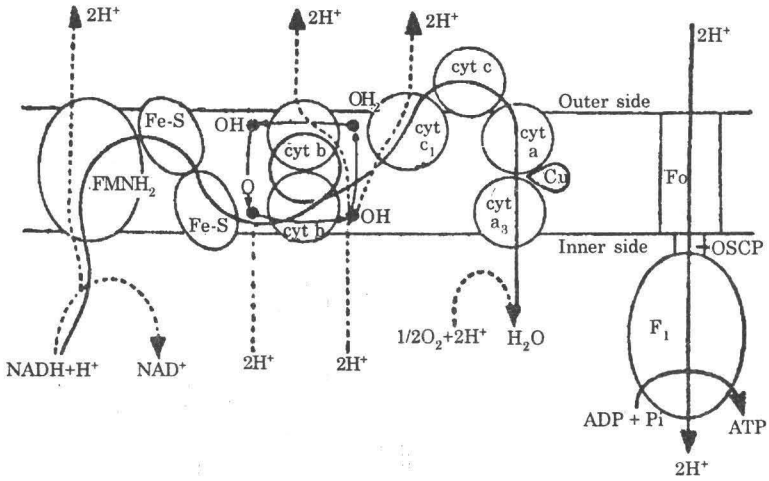


Fig. 24.10. Current view of the location of the enzymes and components of the respiratory chain in the inner membrane of the mitochondrion (After Hinkle McCarty, 1978).

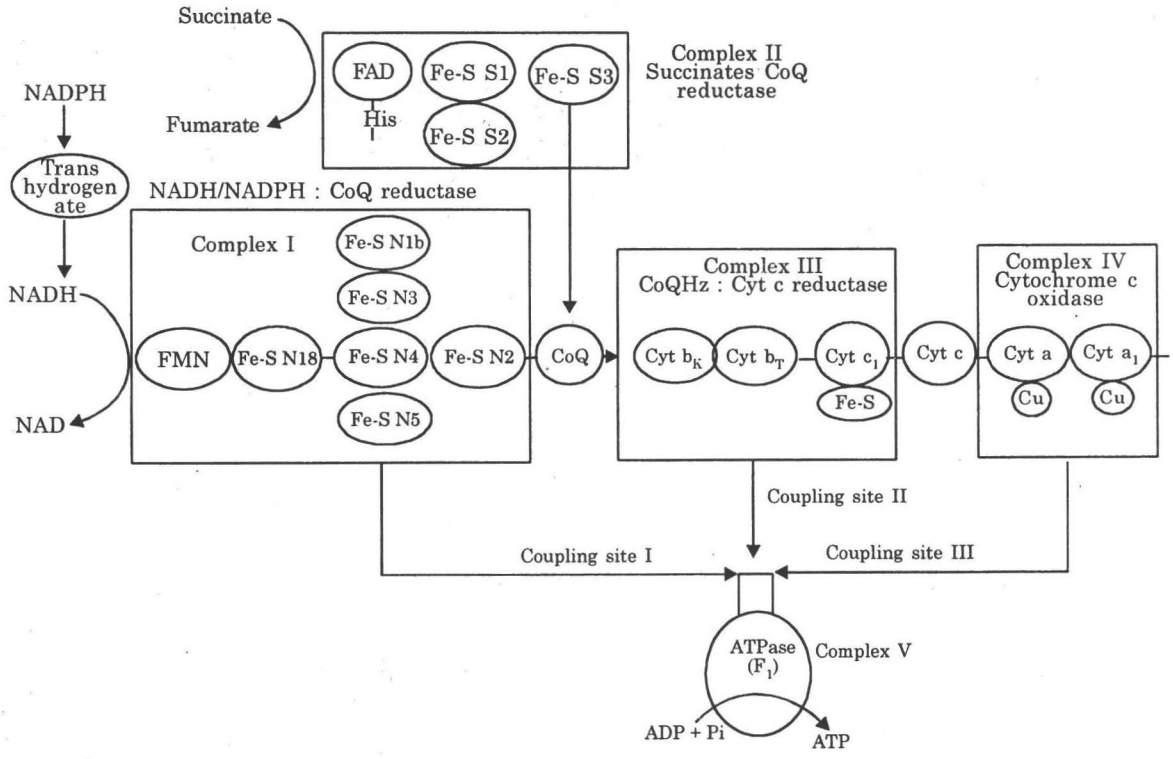


Fig. 24.11. The complexes and components of the mitochondrial respiratory chain. Abbreviations.

ADP—Adenosine diphosphate
ATP—Adenosine triphosphate
ATPase—Adenosine triphosphatase
CoQ—Coenzyme Q=Ubiquinone (UQ)
Cyt—Cytochrome
FAD—Flavin adenine dinucleotide
FMN—Flavin Mononucleotide
Fe-S—Iron-sulphur centres
Hist—Histidine
NAD—Nicotinamide adenine dinucleotide
NADH—Reduced NAD
OSCP—Oligomycin sensitivity conferring protein

Respiratory Chain Complex (Fig. 24.11)

The respiratory chain consists of a series of proteins containing oxidation-reduction groups. The chain is located in the inner membrane of the mitochondrion. Chemical treatment of the mitochondrial membrane results in the isolation of five complexes which have been designated as complexes I, II, III, IV and V (see Hatefi, 1976). The enzymes of the inner membrane of the mitochondria appear to exist as components of these five complexes (Fig. 24.11). Four of the complexes (I, II, III and IV) are parts of the *electron transport system,* while the fifth is concerned with the *conservation and transfer of energy* and *synthesis of* ATP. Two electron carriers, *coenzyme* Q (CoQ) or *ubiquinone* (UQ) and *cytochrome c* can be easily removed from the respiratory chain, and have been called *mobile carriers.* It has been suggested that coenzyme Q acts as a mobile

electron carrier between complexes I and III and II and III, and cytochrome *c* between complexes III and IV. Some workers include both coenzyme Q and cytochrome *c* in complex III. Alternative names for the five complexes are given below :

Complex I— NADH/NADPH : CoQ reductase.

Complex II—Succinate : CoQ reductase.

Complex III— Reduced CoQ ($CoQH_2$) : cytochrome *c* reductase.

Complex IV—Cytochrome *c* oxidase

Complex V—ATPase (ATP synthesizing system).

Complexes I and II are *dehydrogenase complexes* (DH). Pairs of hydrogenes released during aerobic glycolysis and three dehydrogenation reactions of the Krebs cycle are accepted by NAD, which is reduced to $NADH_2$. This compound serves as an energy donor for the respiratory chain and passes hydrogens to complex I. In the fourth dehydrogenation reaction of the Krebs cycle (succinate to fumarate) the hydrogens released are accepted by complex II. The pathway of electrons during oxidation of NADH is complexes I, III and IV. In the oxidation of succinate the pathway is complexes II, III and IV. Thus complexes I and II are alternates. Hydrogens from complexes I and II are accepted by the mobile carrier *coenzyme Q* and transferred to complex III. Electrons are transferred from complex III to complex IV through the mobile carrier *cytochrome.*

Three molecules of ATP are generated per pair of hydrogens/electrons passing down the respiratory chain (one each through complexes I, III and IV, which are energy coupling sites). Here energy released during electron transport is conserved and utilized for synthesis of ATP. ATP generation takes place in complex V. Complex II is a dummy as far as ATP generation is concerned.

1. Complex I (NADH/NADPH : CoQ reductase)

NADH : CoQ *reductase* or NADH *dehydrogenase* contains *flavoprotein* and *iron-sulphur (Fe-S) protein* as its major components. Flavoprotein contains tightly bound *flavin mononucleotide* (FMN) as its prosthetic group. Ragan (1976) has shown that NADH: CoQ reductase of beef heart mitochondria contains at least 16 polypeptides. Of these two (MW 53,000 and 26,000) are parts of flavoprotein, and three (MW 75,000, 53,000 and 29,000), along with minor components, belong to the Fe-S protein region. In addition there is a *proton translocating component* (MW 33,000) spanning the membrane.

Complex I contains FMN and 5–6 iron-sulphur (Fe-S) centres. The iron is not a part of a heme group and is therefore called *non-heme-iron* (NHI). The iron-sulphur centres have been designated as Fe-S Nla, Fe-S Nlb, Fe-S F2, Fe-S N3, Fe-S N4 and Fe-S N5. The N indicates that the centre is a part of NADH dehydrogenase. As with other complexes of the respiratory chain, the lipids of complex I are essentially *phosphatidylcholine* (PC), *phosphatidylethanolamine* (PC) and *cardiolipin.* Complex I catalyses the transfer of electrons from NADH to coenzyme Q (CoQ).

The NADH oxidizing site of complex I is on the M-side (matrix side) of the membrane. The Fe-S Ni and Fe-S N2 centres span the membrane, according to one view. According to another view the Fe-S centre is located in the middle of the membrane.

2. Complex II (succinate-CoQ reductase)

Succinate : CoQ reductase (MW 97,000) consists of two polypeptides, *succinate dehydrogenase* and *Fe-S protein* (Fe-S S3). *Succinate dehydrogenase* has a molecular weight of 70,000 and contains covalently bound FAD and two Fe-S centres (Fe-S S1 and Fe-S S2). The *Fe-S protein* 3 has a molecular weight of 27,000. It contains iron and sulphide but no flavin. Thus complex II contains three iron-sulphur centres, Fe-S S1, Fe-S S2 and Fe-S S3. Succinate dehydrogenase

has its succinate binding site on the M side of the inner mitochondrial membrane. The three Fe-S centres are on the M-side, and there is a close interaction between Fe-S centre 3 and CoQ.

Cytochrome b has also been reported in complex II. This cytochrome $b_{557.5}$ is different from the b-type cytochromes of complex III. It might be point of entry for some unknown branch of the respiratory chain, or might serve as an electron donor for some synthetic or hydroxylation process. Since cyt $b_{557.5}$ can reduce fumarate, it might be involved in electron transfer to *fumarate* in anaerobes.

Complex II catalyses the oxidation of succinate by CoQ. Complex II differs from complex I in that it apparently does not have the ability to translocate protons across the inner membrane.

3. Coenzyme Q (CoQ.Q) or Ubiquinone (UQ)

Conenzyme Q and cytochrome *c* have been called *mobile carriers*. It has been suggested that CoQ acts as a mobile carrier between complexes I and III and II and III. CoQ accepts electrons from $FMNH_2$ of NADH dehydrogenase and $FADH_2$ of succinic dehydrogenase and is reduced to $CoQH_2$ (*hydroquinone* or *dihydroquinone*)

$$FMNH + CoQ \longrightarrow FMN + CoQH_2$$
$$FADH_2 + CoQ \longrightarrow FAD + CoQH_2$$

CoQ is found in mitochondria in the oxidized *quinone* form under anaerobic conditions and in the reduced quinol form under aerobic conditions. The reduced form is called *hydroquinone* or *dihydroquinone.*

Coenzyme Q has a *polyisoprenoid* side chain, which is also found in vitamin K, Vitamin E and plastoquinone of chloroplasts. The number of isoprenoid units varies from 6 to 12 in coenzyme (coenzyme Q_6 ... coenzyme Q_{12}). In mammals the most common form is CoQ_{10}.

It has been suggested that during re-oxidation there is a *semiquinone* (CoQH) stage between $CoQH_2$ and CoQ.

CoQ shares with cytochrome *c* the property of being easily removable from the respiratory chain. It has been suggested that CoQ is located in the hydrophobic middle part of the inner mitochondrial membrane.

4. Complex III ($CoQH_2$ cytochorme *c* reductase)

Complex III has a molecular weight of about 300,000. It consists of proteins having a combined molecular weight of about 240,000 plus bound lipid. Complex III contains at least four centres, two cytochromes *b* (possibly even three), one Fe-S protein and cytochrome c_1. In addition there is an '*antimycin binding protein* and two different species of '*core proteins.*'

The two-*b* type cytochromes are b_T (Cyt. b_{565}) and b_k (Cyt. b_{560}). The letters T and K stand for *transducing* cytochrome *b* and *Keilin type* cytochrome *b*, respectively. The molecular weights of the two *b*-type cytochromes are 37,000 and 17,000. According to one suggestion the cytochromes *b* are located in the middle of the inner membrane. Alternatively it has been suggested that the two-*b*-type cytochromes span the membrane. A third chromophore (Chr_{558}) with a cytochrome-like absorption peak at 558 mm at 77°K has also been reported. It has been suggested that at saturating levels of substrates all the three cytochromes participate in coupled electron transfer. However, at low levels of substrates cyt b_k is reduced but not cyt b_T and Chr_{558}. Therefore electron flow slows down.

Complex III contains a *non-heme iron protein* (MW 26,000) which has the iron-sulphur (Fe-S) centre.

Cytochrome c_1 (cty c_1) from beef heart mitochondria has a molecular weight of 30,600 and is an intrinsic protein. The heme containing part of cyt c_1, which transfers electrons to cytochrome c, is located on the C-side of the membrane. Complex III catalyses the reduction of cytochrome *c* by reduced coenzyme Q ($CoQH_2$).

5. Cytochrome *c* (cyt *c*)

Cytochrome *c* is a peripheral protein located on the C-side (intracristal side) of the inner mitochondrial membrane. Experimental evidence indicates that it remains bound to complex IV (cytochrome *c* oxidase) while functioning in electron transport. Cytochrome *c* consists of a polypeptide chain folded around a heme moiety to which it is bound. The number of amino acid residues in different cytochromes c varies from ~ 85 to ~ 135.

6. Complex IV (cytochrome *c* oxidase)

Cytochrome *c* oxidase is an integral *homoprotein* with a molecular weight of about 200,000. It contains two *heme* and two *copper* centres. The two hemes are *heme a,* which does not react with carbon monoxide, and *heme* a_3, which does. Because of this fact some workers have suggested that cytochrome *c* oxidase consists of two different cytochromes, *cytochrome a* and *cytochrome* a_3. The two have, however, never been separated, and therefore heme *a* and heme a_3 must be considered to be parts of the same complex.

Beef heart cytochrome c oxidase is made up of six polypeptides or subunits (I-VI) with molecular weights of 40,000, 22,500, 15,000, 11,200, 9,800 and 7,300 (Rubin and Tzagoloff, 1973). The yeast enzyme has seven subunits with molecular weights of 40,000, 33,000,22,000, 14,500, 12,700 and 4,600 (Poyton and Schatz, 1975).

According to the evidence available, cytochrome c oxidase is exposed at both the matrix and intracristal sides of the inner mitochondrial membrane. It is thus likely that the enzyme spans the membrane completely. Cytochrome *a* is localized on the C-side of the membrane where it interacts with cytochrome *c*. Cytochrome a_3 is localised on the M-side where it picks up molecular oxygen.

The topology of the subunits of cytochrome c oxidase of beef heart mitochondria has been established by Eytan *et al.* (1975). According to them cytochrome *c* oxidase consists of six subunits, of which subunits II, V and VI are located on the C-side of the membrane, I and IV in the middle, and III on the M-side.

7. Complex V (ATPase complex) (Figs. 24.12 and 24.13)

Electron micrographs of the mitochondrial membranes showed the presence of rounded stalked particles on the matrix side of the inner membrane (Fernandez-Maroon, 1962). These particles have been called *elementary particles* or the *particles of Fernandez-Moran.* Each particle was described as having a *basepiece,* a *stalk* and a *headpiece.* It has been shown that *complex V* or the *ATPase complex* is identical with the particle of Fernandez-Moran.

There are at least 4 coupling factors (F_1, F_2, F_5 (OSCP) and F_6 (Fe_2)] on the M-side.

The *headpiece,* which was identified as the *coupling factor* 1 (F_1) by Racker (1965) is the *ATPase proper.* The F_1 particle has a diameter of 80-100A and a molecular weight of 360,000. Treatment with *urea, NaBr* or *cardiolipin* removes F_1, leaving the stalk. F_1 contains five types of subunits : α (MW 53,000), β (MW 50,000), γ (MW 33,000), δ (MW 17,700) and (MW 7,500). Earlier it was suggested that the number of subunit in F_1 of beef heart mitochondria was 3α, 3β, 1γ, 1δ and 1ε (Senior, 1973). Later, however, this number has been modified as 2α, 2β, 2γ and 2ε, (Senior, 1975). In addition there is an *ATPase inhibitor protein* (1) (MW, 10,000) which can be removed by *trypsin.*

The *stalk* portion may be F_5 or *oligomycin-sensitivity conferrring protein* (OSCP) and F_6 (Fc_2). McLennan and Asai (1968) showed that purified OSCP has measurements similar to those of the stalk. OSCP is necessary for binding of F_1 to the inner mitochondrial membrane. Treatment with *ammonia* releases OSCP. Fc_2 or F_6 is also required for binding F_1 to the membrane. It has a molecular weight of 8,000 and can be removed by treatment with *silicotungstate.*

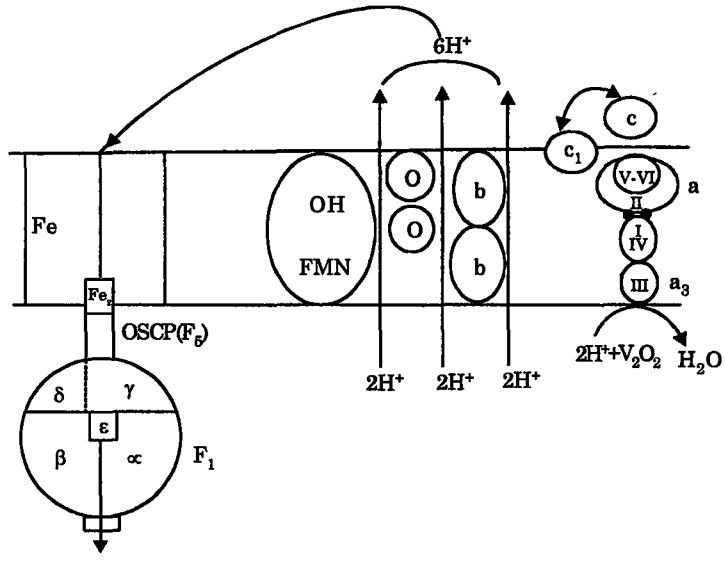


Fig. 24.12. Components of the respiratory chain and ATPase (modified from Racker, 1977 and DePierre and Ernster, 1977). DH-Dehydrogenase complex. I-VI Subunits of cytochrome oxidase (a. a_3).

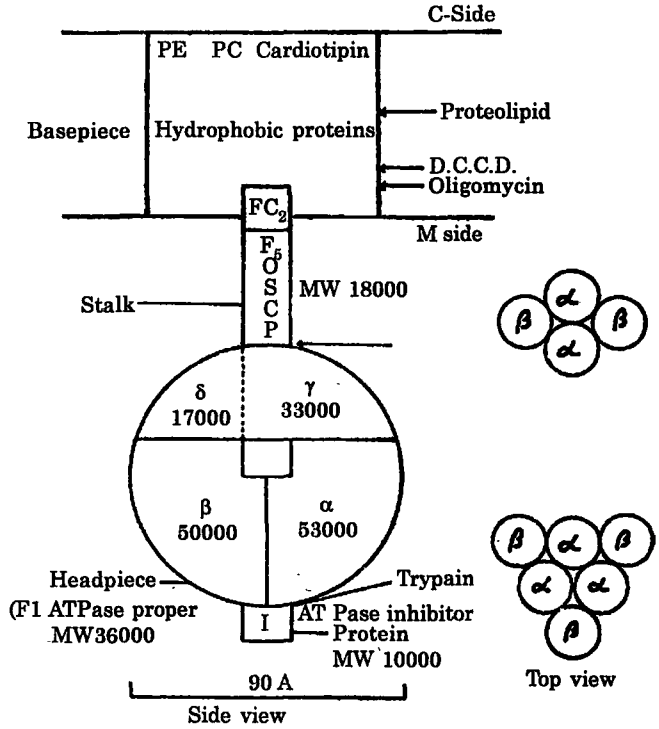


Fig. 24.13. Complex V, ATP synthesizing system.
DCCD—Dicyclohexyl carbodimide.
F_1—ATPase proper (headpiece).
F_5 (OSCP)—oligomycin sensitivity conferring protein.
F_6 (FC_2)—required for binding F_1 to membrane,
I—ATP ase inhibitor protein.
α, β, γ, δ, ε—subunits of F_1.
The possible configurations of α and β subunits are shown on the right.

The *basepiece* is the portion within the inner mitochondrial membrane. It has been isolated as F_0 and provides the *proton channel.* It is composed of a group of hydrophobic proteins and phospholipids (*'proteolipid'*). The ATPase complex is sensitive to inhibitors like *oligomycin* and *dicyclohexyl carbodimide* (DCCD). The proteolipid contains binding sites for both oligomycin and DCCD. The membrane sector of the ATPase complex probably contains the proton translocating system of the enzyme.

The lipids present in the inner mitochondrial membrane are essentially *phosphatidylcholine* (PC), *phosphatidylethanolamine* (PE) and *cardiolipin.*

Table 24.2

COMPONENTS OF THE RESPIRATORY CHAIN

A. Complex I (NADH : CoQ reductase). MW 850,000

1. NADH dehydrogenase—Flavoprotein with flavin mononucleotide (FMN) as the prosthetic group. Protein portion is a single polypeptide chain. MW 70,000.

2. Non-heme iron proteins (NHI) with iron-sulphur centres (Fe-S). There are 6 Fe-S centres : Fe-S Nla, Fe-S Nlb, Fe-S-N2, Fe-S N3, Fe-S N4 and Fe-S-N5.

B. Complex II (Succinate : CoQ reductase) MW 970,000

1. Succinate dehydrogenase : MW 70,000

Contains covalently bound FAD as the prosthetic group and two Fe-S centres (Fe-S S1 and Fe-S S2).

2. Fe-SS3 protein. MW 27,000.

3. Cytochrome *b* absorbance 557.5 mm.

C. Coenzyme Q (CoQ) or ubiquinone (UQ). Mobile carrier between complexes I and II and III.

D. Complex III ($CoQH_3$: cyt *c* reductase) MW 280,000,

1. Cytochrome b_K. MW 30,000. Absorbance 560 mm.

2. Cytochrome b_T. MW 50,000. Absorbance 565 nm.

3. Cytochrome c_t. consists of polypeptides.

(*a*) MW 29,000 bears heme moiety.

(*b*) MW 15,000

4. Non-heme iron protein with iron sulphur (Fe-S) centre MW 26,000

5. core proteins.

6. Antimycin-binding protein.

E. Cytochrome *c* mobile carrier between complex III and IV. MW~13,000.

Contains 1 *c*-heme bound to polypeptide chain of ~85 to ~135 amino acids.

F. Complex IV (Cytochrome c oxydase). MW 200,000. Composed of cytochromes *a* and a_3 and 2 atoms of Cu.

1. Cyt *a*-not inhibited by CO.
2. cyt a_3-inhibited by CO.
3. Cuα
4. Cuβ

Beef heart oxidase consists of 6 subunits (polypeptides).

Subunit I—MW 40,000—in middle of membrane.

Subunit II—MW 22,500—on C-side of membrane.

Subunit III—MW 15,000—on M-side of membrane.

Subunit IV—MW 11,200—in middle of membrane.

Subunit V—MW 9,800—on C-side of membrane.

Subunit VI—MW 7,300—C-side of membrane.

G. Complex V (ATPase) complex

Consists of headpiece, stalk and basepiece.

1. Headpiece (F_1) MW 360,000 consists of 5 subunits + inhibitor.

α (A) subunits—2 or 3. MW 53,000

β (B) subunits—2 or 3. MW 50,000

γ (C) subunits—1 or 2. MW 33,000

δ (D) subunits—1 or 2. MW 1,75,000

ε (E) subunits—1 or 2. MW 7,000

F_1 inhibitor protein I—MW 10,000

2. Stalk

F_5 or ligomycin sensitivity conferring protein (OSCP). MW 18,000.

F_6 or Fc_2—MW 8,000.

3. Basepiece : (Fo) ("proteolipid"). Hydrophobic protein complex contains proton channel.

Proteins :	MW	29,000
	MW	22,000
	MW	12,000
	MW	7,8000

Location of the electron transport and phosphorylating systems : The components of the *electron transport system* and the *phosphorylating system* are located in the inner membrane of the mitochondrion. The side of this membrane facing the matrix is called the M-*side* while the side facing the intermembrane space is called the *C-side.*

Complexes I and II : The flavin components of the respiratory chain are on the M-side. Harmon and Crane (1976) have suggested that both NADH : CoQ *reductase* (complex I) and *succinate : CoQ reductase* (complex II) span the membrane in beef heart mitochondria. The $NADH^+$ oxidizing site of complex I is on the M-side. Similarly the succinate binding site of succinate-CoQ reductase is on the M-side. Mitchell (1972) and Gutman *et al.* (1975) have proposed that the Fe-S centres 1 and 2 of complex I span the membrane (Fig. 24.14). According to topological evidence, however, complex I does not seem to form a redox loop across the membrane and the Fe-S centre is the middle of the membrane (Fig. 24.15). The three Fe-S centres of complex II appear to be located on the M-side.

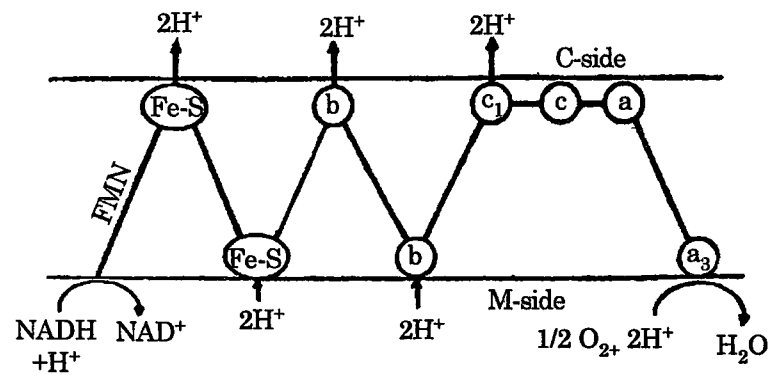


Fig. 24.14. Redox loops (Mitchell, 1976), Complexes I, III and IV.

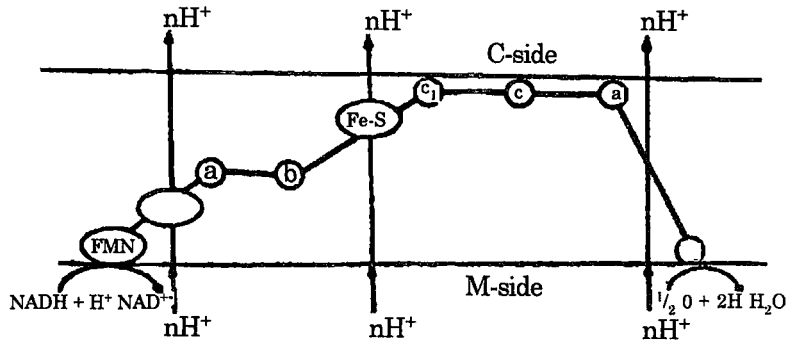


Fig. 24.15. Location of the components of the respiratory chain (DePierre and Ernster 1977).

Coenzyme Q : According to one view, it is located on the C-side and can move across the membrane. According to another view it could be localized in the hydrophobic middle of the membrane.

Complex III : The two *b*-type cytochromes either span the inner mitochondrial membrane or are located in the middle of the lipid bilayer. Cytochromes c_1 and *c* are located in the C-side.

Complex IV : It is likely that cytochromes a-a_3 span the membrane, with cytochrome *a* on the C-side and cytochrome a_3 on the M-side.

Complex V : The ATPase complex or complex V consists of a *basepiece* (F_0) located within the membrane, a *stalk* consisting of coupling factors Fc_2 (F_6) and OSCP (F_5), and a *headpiece* consisting of the ATPase proper (F_1) on the M-side.

The formation of ATP from ADP and Pi is coupled to electron transport in the inner mitochondrial membrane.

Electron Transport Mechanism

1. H atoms : A hydrogen atom consists of one proton and one electron. Protons are soluble in the water of the cell, but electrons are not. According to the chemiosmotic hypothesis hydrogen carriers alternate with electron carriers. When an electron carrier interacts with a hydrogen atom, it accepts only the electron. The proton is released into the aqueous solution of the cell.

2. NAD^+ : Hydrogen atoms liberated in the dehydrogenation reaction of aerobic glycolysis, oxidation of pyruvic acid and the Krebs cycle are accepted by *nicotinamide adenine dinucleotide* (NAD^+) (except in one dehydrogenation reaction, succinate to fumarate). The NAD^+ molecule normally carries a positive electric charge, and hence the plus sign. Each molecule of NAD^+ accepts *two electrons and one proton* and is reduced to NADH.

3. FMN : NADH donates two electrons and a proton to *flavin mononucleotide* (FMN), and is oxidized back to NAD^+. FMN accepts the two

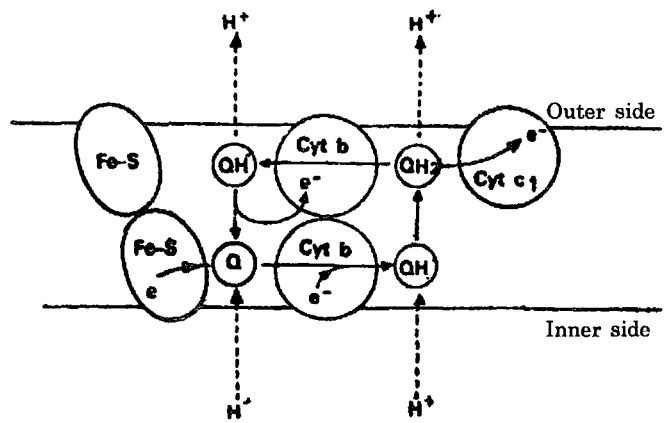


Fig. 24.16. Components of the quinone cycle. Only one CoQ molecule has been shown. Actually the Fe-S protein releases two electrons to two CoQ (UQ.Q) molecules.

electrons and the proton from NADH and *another proton from the internal medium,* and is reduced to $FMNH_2$.

4. Fe-S : $FMNH_2$ now gives up two protons and two electrons and is oxidized to FMN. The two electrons are acquired by the iron-sulphur (Fe-S) proteins, which, however, cannot accept the protons. *The two protons are transported outside the mitochondrion.*

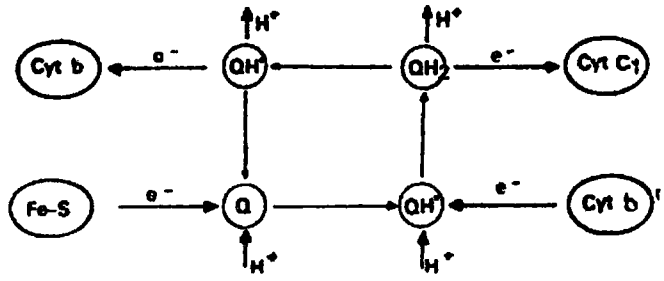


Fig. 24.17. The quinone cycle.
Q — Fully oxidized quinone
QH — Semiquinone
QH_2 — Hydroquinone.

5. The quinone cycle (Figs. 24.16 and 24.17). The iron sulphur proteins donate the pair of electrons to *ubiquinone* (UQ), also called *coenzyme Q* (CoQ, Q). Ubiquinone can exist in three possible states of oxidation, *quinone, semiquinone* and *hydroquinone.*

(i) Quinone (Q) is the fully oxidized form, with two oxygen atoms connected to the ring by double bonds.

(ii) Semiquinone (QH) is the semi-reduced form of ubiquinone, with a hydrogen atom attached to one oxygen.

(iii) Hydroquinone (QH_2) is the fully reduced form, with hydrogen atoms attached to both oxygens.

According to the *Q cycle* proposed by Mitchell, ubiquinone undergoes changes in the oxidation state during electron transfer. Ubiquinone is considered to be a *mobile carrier* which migrates from one side of the membrane to the other. It is assumed that two molecule of ubiquinone are involved in the cycle.

(a) Fe-S protein donate one electron to each of two quinone (Q) molecules. The molecules *take up a proton* (H^+) *each from the internal medium,* and two molecules of *semiquinone* (QH) are formed.

(b) Each QH receives one electron from cytochrome b and one proton from the interior of the mitochondrion to form hydroquinone (QH_2).

(c) It is assumed that the two hydroquinone molecules, with a total of four protons (H^+) received from the inner surface of the membrane, cross over to the outer surface. Here each QH_2 donates an electron to cytochrome c_1, and *the proton is released outside.* In this process QH_2 is converted to the semireduced QH state.

(d) Each semi-quinone (QH) transfers an electron to cytochrome *b* and a proton to the external medium, and becomes fully oxidized to quinone (Q). *A total of four protons are transported outside the mitochondrion during the Q cycle.*

The 'protomotive Q cycle' was proposed by Mitchell (1975-1976). The role of Q as an obligatory redox carrier between cytochromes *b* and c_1 is, however, not supported by some studies. The vectorial movement of Q and QH_2 across the membrane is not likely to occur (De Pierre and Ernster, 1977).

6. Cytochrome *c* oxidase (cyt *a*. a_3 2 C_u)

Coenzyme Q is a *hydrogen carrier,* while the cytochromes are *electron carriers.* After coenzyme Q, the hydrogens are split up into electrons and protons. The electrons pass down cytochromes *b*

and *c* and are accepted by cytochrome *c* oxidase. The protons are released into the aqueous environment.

Cytochrome *c* oxidase contains 4 units (cyt *a*, cyt a_3, Cuα and Cuβ) which can carry one electron each. Thus 4 electrons are stored in the enzyme prior to discharge. The 4 electrons ($4e^-$) combine with 4 protons ($4H^+$) to form the aqueous medium and one molecule of oxygen to form 2 molecules of water.

$$4H^+ + 4e^- + O_2 = 2H_2O$$

Nicholls has suggested two mechanisms, a *sequential mechanism* and a *quasi-concerted mechanism* to explain the action for cytochrome *c* oxidase. According to the *sequential mechanism* the four electron carriers (cyt *a*, a_3, Cuα, Cuβ) form a chain between cytochrome c and oxygen. Each component carries one electron at a time.

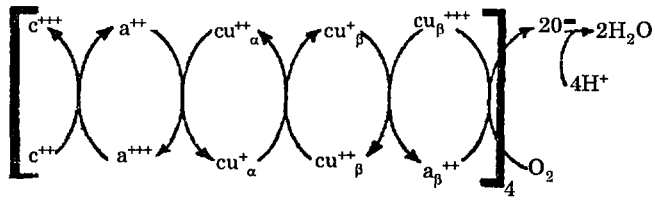


Fig. 24.18. Sequential reaction mechanism for cytochrome oxidase.

In the *quasi-concerted mechanism,* the four carriers constitute a 4 electron sink and are reduced simultaneously.

The iron atoms in cytochromes a and s_3 alternate between the ferric (Fe^{3+}) and the ferrous (Fe^{2+}) states during electron transport. The copper atoms alternate between Cu^{2+} and Cu^+ states.

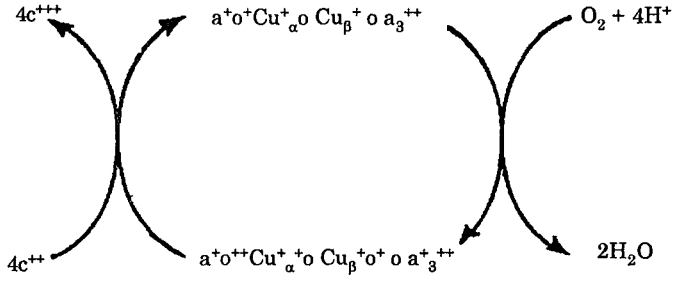


Fig. 24.19. Quasi-concerted reaction mechanism for cytochrome oxidase.

7. Oxidative phosphorylation : It will be seen that for each pair of electrons transferred from NADH down the respiratory chain to oxygen, *six* protons are translocated across the mitochondrial membrane from the inside to the outside. This increases the proton concentration outside the membrane, and sets up a *proton gradient.* The resulting electric potential forces the protons through complex V (F_0-F_1 complex, ATPase) back into the mitochondrion, and provides energy for the synthesis of ATP. For every pair of protons driven inwards, one molecule of ATP is synthesized from ADP and inorganic phosphate (Pi). Thus for the three pairs of protons passing through the ATPase complex, three molecules of ATP are generated.

Energy Conservation During Oxidative Phosphorylation

The mechanism of coupling between electron transport and ATP synthesis is not yet known. There are three hypotheses regarding the mechanism of oxidative phosphorylation (1) the *chemical coupling* hypothesis, (2) the *chemiosmatic* hypothesis and (3) the *conformation coupling* hypothesis.

(1) Chemical coupling hypothesis : Oxidative phosphorylation was discovered in 1939 and about 15 years later the chemical coupling hypothesis was put forward to explain coupling between the flow of electrons through the electron transfer system and phosphorylation of AFP. Two hypothetical coupling factors (enzymes), X and E were supposed to be involved at each ATP

generating step (Fig. 24.19). It was suggested that the coupling factors were different at each of the three ATP-generating steps (X_1 and E_1, X_2 and E_2, X_3 and E_3).

Coupling factor X first forms a *high energy intermediate complex* with a respiratory enzyme like cytochrome *b* ($Fe^{3+} \sim X$). In the next step the respiratory enzyme is replaced by inorganic phosphate (PO_4^{3-}) resulting in the formation of a phosphorylated intermediate (X—P) with a high energy phosphate group. The coupling enzyme X is then replaced with enzyme E to form E—P. This enzyme catalyses the formation of ATP from ADP and phosphate. The presumed function of the coupling factors X and E is, therefore, to transfer the energy released in the redox reaction for ATP synthesis.

An extensive search has been made for the coupling agents and the high-energy intermediates, but none have been found. The explanation given for their not being located is that they are extremely unstable. But it has also been suggested that the coupling factors have not been found because they do not exist. The chemical coupling hypothesis is only of historical importance today.

(2) The chemiosmotic hypothesis : In 1961 Mitchell proposed the chemiosmotic hypothesis according to which the *electron transfer chain* generates an electrochemical *proton concentration gradient* across the membrane, and that this gradient is the driving force for *synthesis of ATP.* The initial theory has been modified subsequently, and the essential features of the chemiosmotic hypothesis as it stands today are given below.

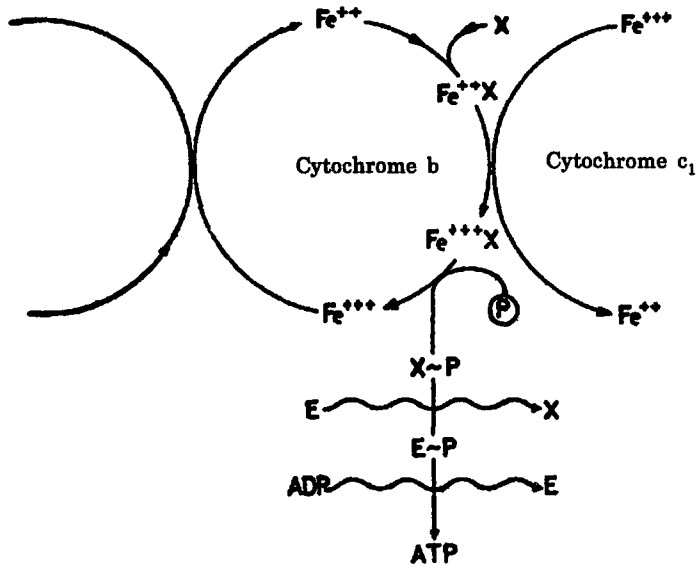


Fig. 24.20. The chemical coupling hypothesis of oxidative phosphorylation.

(i) There are at least three kinds of flow in the inner mitochondrial membrane (Fig. 24.21).

(a) Electron transport (J_0)

(b) Proton translocation (J_H).

(c) ATP synthesis (J_p)

(ii) The electron transport system is located within the inner mitochondrial membrane, and the *phosphorylating system* in the knoblike F_1 particles (headpieces).

(iii) It is assumed that ATP synthesis (j_p) and electron transport (J_0) are coupled through proton translocation (J_H) (Fig. 24.21).

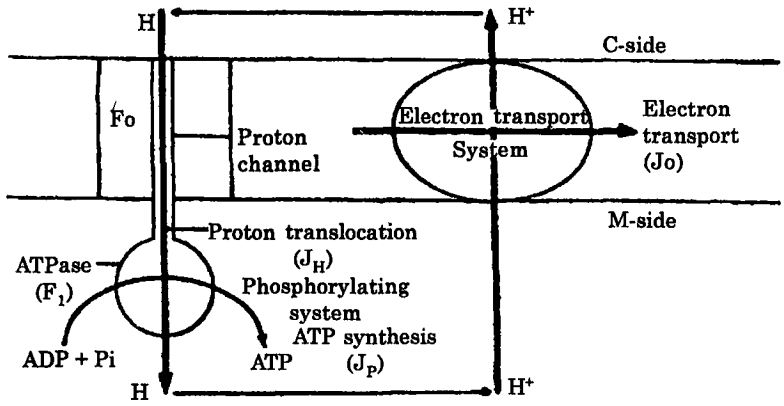


Fig. 24.21. Chemiosmotic hypothesis. The three flows (j_O, J_H, H_P) through the inner mitochondrial membrane.

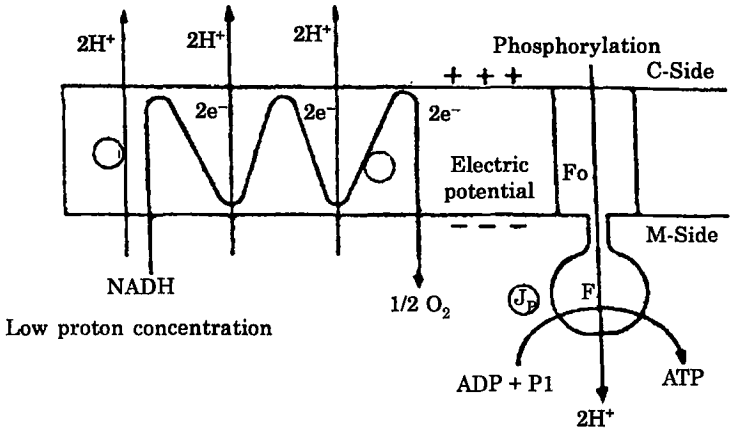


Fig. 24.22. Chemiosmotic hypothesis. ATP synthesis (J_P) and electron transport (J_O) are coupled through proton translocation (J_H).

(iv) Certain stages of electron transport involve *liberation* of hydrogen ions (protons), *e.g.*,

$$\text{Malate} + NAD^+ \longrightarrow \text{Oxaloacetate} + NADH + H^+$$

$$CoQH_2 + 2\ \text{Cyt}\ b\ (Fe^{3+}) \longrightarrow CoQ + 2\ \text{Cyt}\ b\ (Fe^{2+}) + 2H^+$$

(v) Other stages of electron transport involve *uptake* of hydrogen ions, *e.g.* :

$$NADH + H^+ + FP \longrightarrow NAD^+ + FPH_2$$

$$2\ \text{Cyt}\ a\ (Fe^{2+}) + 2H^+ + 1/2\ O_2 \longrightarrow 2\ \text{Cyt}\ a_3\ (Fe^{3+}) + H_2O$$

(vi) It is assumed that steps involving hydrogen ion *uptake* take place on the inner side (M-side) of the mitochondrial membrane, and those involving *liberation* of hydrogen ions on the outer side (C-side).

(vii) The electron transport carriers are so arranged in the membrane that electron transfer from centres of low redox potentials to high redox potentials is coupled with transport of protons accross the membrane. In other words the electron transport system operates a '*proton pump*' which transports protons (H^+) from the inside to the outside of the mitochondrial membrane. This process is called *proton translocation. The* protons are transported outside at high electrochemical potential (*Protonmotive force*).

(viii) Transport of protons to the outside of the membrane causes positive charges to accumulate outside the membrane, *i.e.*, a *proton concentration gradient* or *membrane potential* is created across the membrane.

(ix) The proton concentration gradient tends to force the protons back from outside to inside. The energy of the gradient drives the process of ATP synthesis.

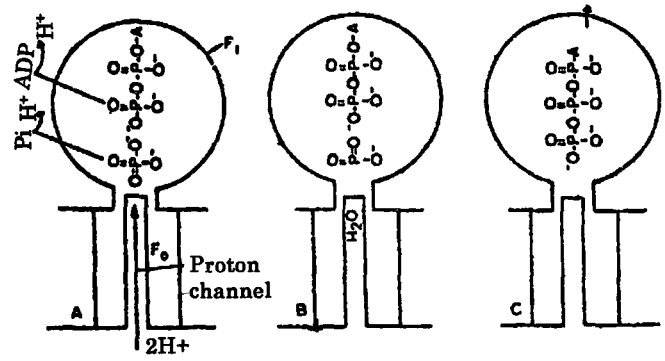


Fig. 24.23. Phosphorylation mechanism according to Mitchell.

A. Two protons move through the proton channel in F_0 and attack one of the oxygens of phosphate.

B. The oxygen is pulled loose and removed as a molecule of water.

C. The oxygen of ADP reacts with the phosphorus atom of the phosphate to form ATP, which then dissociates from ATPase (F_1).

(x) In general, the inner mitochondrial membrane is impermeable to hydrogen ions. Thus the back flow of protons can take place only through special regions, the *proton channels*, in the ATPase system. The ATPase system consists of the F_0-F_1 *complex* (formerly called the *elementary particles*). F_0 is within the membrane (basepiece) and serves as a *proton channel*, F_1 (ATPase proper, headpiece) is the active site of ATP synthesis.

(xi) The passage of protons through the F_1 particles (ATPase) is utilized for the *synthesis of ATP* from ADP and inorganic phosphate (P_i). One molecule of ATP is generated for every two protons passing through F_0-F_1 complex. The direct phosphorylation mechanism proposed by Mitchell is explained in Figure 24.22.

(xii) Hydrogen ions (OH^-) are moved outward and H_2O dissociates into OH^- and H^+ to replace them. The hydroxy ions combine with free H^+ to form water. Thus OH^- efflux is essentially similar to H^+ influx.

(3) The conformational coupling hypothesis : It has been found that the membranes of the cristae assume different forms during different functional states of the mitochondrion. In the *non-energized* state of the mitochondrion, under conditions of lack of energy supply, the cristae are in the form of straight flattened sacs. The stalk of the repeating units is contracted, and the headpiece is flattended into a disc (Fig. 24.24). When substrates are given to isolated mitochondria or when mitochondria are kept in a solution containing ATP, they go into the *energized* state. The cristae become more organized and assume a vesicular form. Repeating units assume an extended form and are dumb bell-shaped. If inorganic phosphate (without ADP) is added to mitochondria, the membranes of the cristae become convoluted and assume a zig-zag shape. The repeating units become spherical.

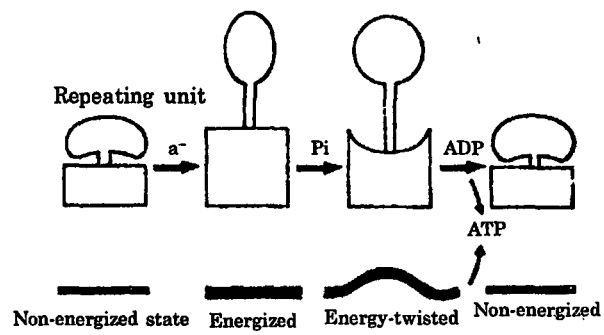


Fig. 24.24. Conformational coupling hypothesis.

Addition of ADP brings about change from the energy-twisted to the energized

state. Addition of uncouplers or cations that can be transported across the membrane can change the energized state to a non-energized one.

The changes described above indicate the presence of a conformational cycle of repeating units during coupled reactions. The twisted form stores the energy released during electron transport. When energy is required for ATP synthesis the energy-twisted or energized state returns to the stable non-energized state. The energy released is utilized for the synthesis of ATP from ADP and Pi.

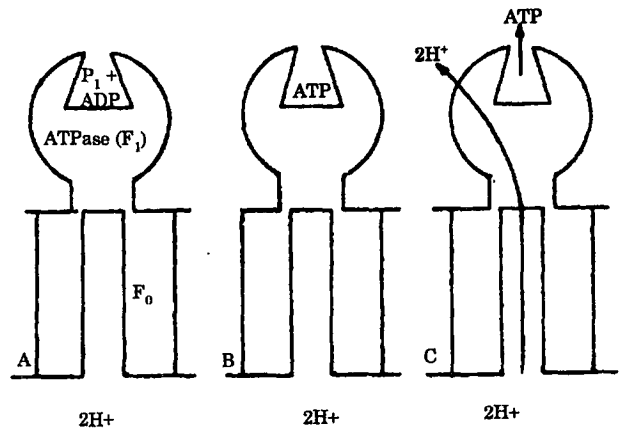


Fig. 24.25. Conformational change mechanism (modified Boyer),

A. ADP and Pi combine spontaneously in active site of ATPase.

B. ATP formed is tightly bound to ATPase.

C. Energy supplied by protons causes conformational change of ATPase, releasing ATP.

The original conformational hypothesis was proposed by Boyer (1965). According to this hypothesis (there is a direct communication between the electron transfer catalysts and the ATP-synthesizing components through polypeptide-polypeptide interaction. A more recent formulation of this hypothesis (1974) has been called the *Boyer-Slater conformational coupling hypothesis.* In the modified hypothesis, electron transfer is believed to induce conformational changes leading to translocation of protons. Conformational changes in the electron transfer proteins induce changes in the ATP synthesizing protein components.

In the conformation hypothesis the protons are believed to have a less direct role than in the chemiosmotic hypothesis. The central idea of this hypothesis is that passage of protons through F_1 could change the conformation of its proteins. These proton-induced conformational changes near the active site could result in the synthesis of ATP.

ADP and inorganic phosphate (Pl) could combine to form ATP at the active site of F_1 without requiring free energy (Fig. 24.25 and 24.26). Energy would, however, be required to release the tightly bound ATP molecule from F_1. Protons binding elsewhere than the active site could cause conformational changes in ATPase (F_1) resulting release of ATP. The protons would then be released into the solution on the M-side of the membrane. The whole scheme can be summarized thus :

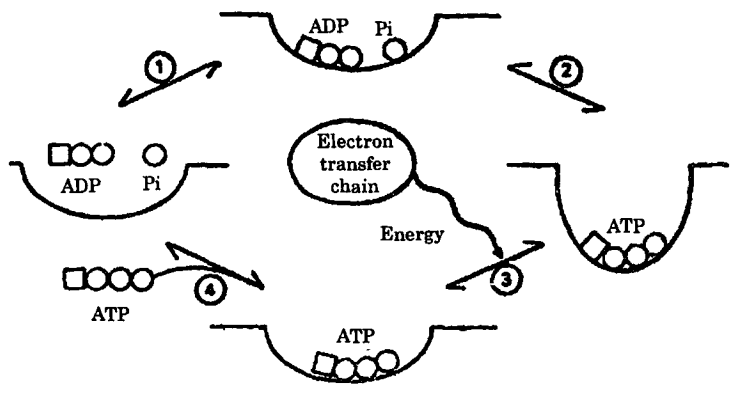


Fig. 24.26. Oxidative phosphorylation, modified Boyer model.

1. ADP and Pi attach to active site of ATPase.
2. ADP + Pi combine spontaneously to form ATP.
3. Energy supplied proton translocation by the electron transfer chain causes conformational change of ATPase.
4. ATP released as a result of conformational change.

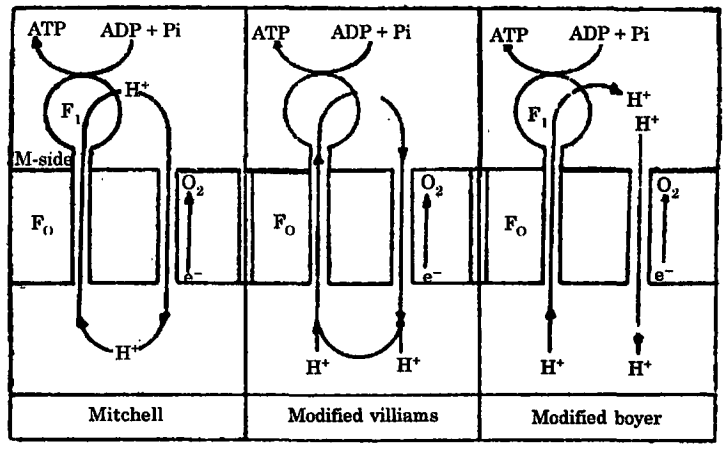


Fig. 24.27. Hypothesis regarding of electron transfer with phosphorylation and proton translocation.

1. ADP + Pi ⟶ ATP (on active site of F_1).

2. Protons bind to F_1 Conformational changes of F_1 ⟶ Release of ATP.

The conformation hypothesis does not affect the central thesis of Mitchell's chemiosmotic hypothesis. Mitchell (1976) himself considered the involvement of conformational changes in chemiosmotic coupling. In fact Mitchell's hypothesis becomes more attractive when coupled with conformational processes.

Williams (1961-1970) believes that the protons produced by the electron transfer chain remain in the membrane (Fig. 24.27). A channel present in the membrane is thought to communicate with both sides of the membrane.

25 Biosynthesis of Nucleic Acids

25.1 Biosynthesis of Purine Nucleotides

De novo synthesis of the purine ring system is a nearly universal biochemical capacity. The precursors of the purine ring were first established in the laboratory of Buchanan by administering labelled isotopes of carbon and nitrogen compounds to pigeons. These studies revealed the following general picture of the origin of the purine nucleus. Carbon atoms 2 and 8 are derived from *formate* or the 1-carbon unit arising from various compounds, *e.g., serine* and *glycine.* Carbon atom 6 is derived from CO_2. Carbon atoms 4 and 5 come from *carboxyl* and *methylene* carbons of *glycine,* respectively. Nitrogen 7 is also derived from *glycine.* It is apparent that glycine is incorporated as a whole. Nitrogen atom 1 is derived from *amino nitrogen of aspartic acid* and nitrogens at position 3 and 9 come from the *amide nitrogen of glutamine* (Fig. 25.1)

A. De novo Synthesis of Inosinic Acid

Inosinic acid is the first product formed with a complete purine ring structure in the biosynthesis of purine nucleotides. The general route for urine biosynthesis has been studied in many species and is essentially the same in all organisms. The detailed biosynthetic pathway, for which the enzymes have now been isolated and studied, is

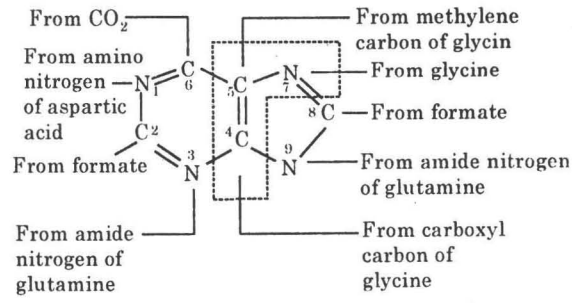


Fig. 25.1. Origin of carbon and nitrogen atoms of the purine ring from different precursors.

indicated in Fig. 25.2. The pathway consists of a series of successive reactions by which the purine ring system is formed on *carbon-1 of ribose 5-phosphate.* This directly leads to the formation of *purine ribonucleotides.* Free purines of nucleosides never appear as intermediates in this pathway.

(a) 5-Phosphoribosyl 1-pyrophosphate (PRPP) : ATP-dependent pyrophosphorylation of ribose 5 phosphate gives *5-phosphoribosyl-1-pyrophosphate.* This compound is a key substance in the biosynthesis of both purine and pyrimidine nucleotides. The reaction is interesting in that it is catalysed by a *kinase* that transfers *pyrophosphate* rather than phosphate from ATP.

$$\text{Ribose-5 phosphate} + \text{ATP} \xrightarrow{Mg^{2+}} \alpha\text{-5-Phosphoribosyl-1-pyrophosphate} + \text{ATP}$$

(b) 5-Phosphoribosyl-1-amine : The second step of this pathway involves the glutamine-dependent amination of PRPP, yielding a labile amino sugar, 5-*phosphoribosyl-1-amine* in presence of *glutamine phosphoribosyl pyrophosphate amido-transferase.* The reaction incorporates a nitrogen atom into position 9 of the purine ring. The amination step results in the inversion of the configuration at carbon 1 to ribose, since *purine ribosides* are of the β configuration at carbon 1 of ribose, since *purine ribosides* are of the β configuration while *5-phosphoribosyl-pyrophosphate* has the α-configuration. Hence, an N-glycoside bond of proper stereochemistry is introduced early in the reaction sequence. This reaction is the "*committed step*" in purine biosynthesis, and is subject to feedback inhibition. The enzyme purified from *A. aerogenes* was inhibited by low concentrations of purine ribonucleotides.

$$\text{PRPP} + \text{Glutamine} \xrightarrow{Mg^{2+}} \text{5-Phosphoribosyl-1-amine} + \text{Glutamic acid} + \text{PPi}$$

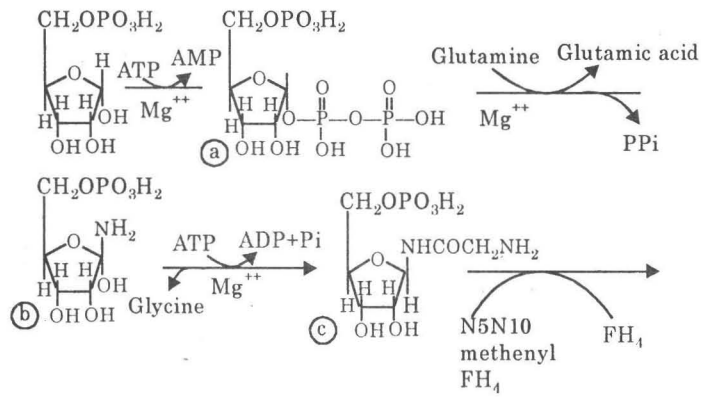
(c) Glycinamide ribonucleotide : The next reaction is catalysed by *phosphoribosyl glycinamide synthetase.* This is an ATP dependent reaction wherein the entire structure of glycine is conjugated through the formation of an amide linkage with 5-phosphoribosyl-1-amine. The enzyme isolated from *A. aerogenes* is not inhibited by purine ribonucleotides.

$$\text{Phosphoribosyl amine} + \text{ATP} + \text{Glycine} \xrightarrow{Mg^{2+}} \text{Glycinamide ribonucleotide} + \text{ADP} + \text{Pi}$$

(d) α-N-formylglycinamidine ribonucleotide : Glycinamide ribonucleotide is formulated to yield α-N-formyleglycinamide ribonucleotide. This reaction is catalysed by *glycinamide ribonucleotide transformylase.*

The enzyme transfer a formyl residue from N^5, N^{10}-methenyltetra-hydrofolate. The latter can be generated from free formate, accounting for the labelling pattern indicated in Fig. 25.1.

Glycinamide ribonucleotide + N^5, N^{10}-methenyltetrahydrofolate + H_2O ⟶ α-N-Formylglycinamide ribonucleotide + Tetrahydrofolate + H^+.



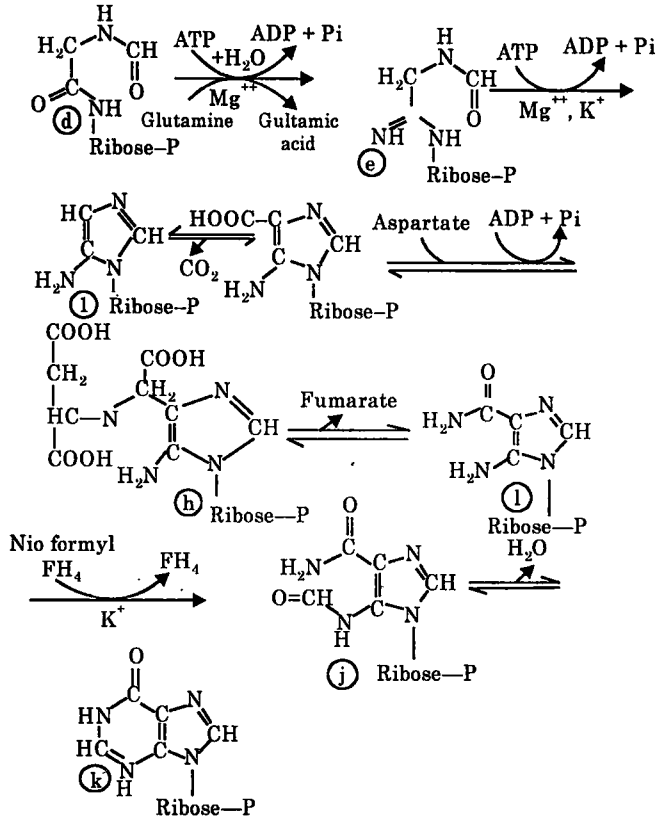


Fig. 25.2. Biosynthesis of inosinic acid from the ribose-5-Phosphate.

In Fig. 25.2, we have

(a) 5-Phosphoribosyl-1-pyrophosphate (PRPP).

(b) 5-Phosphoribosyl-amine.

(c) Glycinamide nucleotide.

(d) α-N-formylglycinamide ribonucleotide.

(e) α-N-formylglycinamidine ribonucleotide.

(f) 5-Aminoimidázole ribonucleotide.

(g) 5-Aminoimidazole-4-carboxylic acid ribonucleotide.

(h) 5-Aminomidazole-4-N-succinocarboxamide ribonucleotide.

(i) 5-Aminoimidazole-4-carboxamide ribonucleotide.

(j) Formamidoimidazole-4-carboxamide ribonucleotide.

(k) Inosinic acid.

(e) α-*N-formylgly cinamidine ribonucleotide :* The next step involves transfer of the amide group from glutamine to α-*N* formylglycinamide ribonucleotide to form α-*N-formylglycinamide ribonucleotide* to form α-*N-formylglycinamide ribonucleotide* to form α-*N-formylglycinamidine ribonucleotide*. This is the second glutamine-dependent amination which inserts the nitrogen atom at position 3 of the purine ring.

α-N-Formylglycinamide ribonucleotide + Glutamine + ATP + H_2O $\xrightarrow{Mg^{2+}}$ α-N-Formylglycinamidine ribonucleotide + Glutamic acid + ADP + Pi

(f) 5-Aminoimidazole ribonucleotide : α-N-Formylglycinamidine ribonucleotide has all the structural features of the imidazole ring of purines. This ring is closed by an ATP dependent

dehydration catalyzed by the enzyme *5'-phosphoribosyl formylgycinamidine cycloligase* to yield *5-aminoimidazole ribonucleotide.*

α-N-Formylglycinamidine ribonucleotide + ATP $\xrightarrow[K^+]{Mg^{2+}}$ 5-Aminoimidazole ribonucleotide + ADP + Pi

(g) 5-Aminoimidazole-4-carboxylic acid ribonucleotide : A molecule of CO_2 is now incorporated through a reaction catalysed by *aminoimidazole ribonucleotide carboxylase* to yield *5-aminoimidazole-4-carboxylic acid ribonucleotide.*

5-Aminoimidazole ribonucleotide + CO_2 $\rightleftharpoons$ 5-Aminoimidazole-4-carboxylic acid ribonucleotide

(h) 5-Aminoimidazole-4-N-succinocarboxamide ribonucletoide : The conversion of 5-aminoimidazole-4-carboxylic acid ribonucleotide to the corresponding amide occurs in a two step process. First, an *amide linkage* between the substrate and aspartic acid is introduced in an ATP dependent reaction catalyzed by the enzyme *5'-phosphoribosyl-4-carboxy-5-aminoimidazole* L *aspartate ligase.* This produces the intermediate *5-aminoimidazole 4*-N-*succinocarboxamide ribonucleotide.*

5-Aminoimidazole-4-carboxylic acid ribonucleotide + Aspartic acid + ATP $\rightleftharpoons^{Mg^{2+}}$ 5-Aminoimidazole-4-N-succinocarboxamide ribonucleotide + ADP + Pi

(i) 5-Aminoimidazole-4-carboxamide ribonucleotide : 5-Aminoimidazole-4-N-succinocarboxamide ribonucleotide subsequently undergoes an *elimination reaction wherein* the carbon skeleton of the aspartate molecule is eliminated as *fumarate.* This inserts the nitrogen atom *at position 1 of the purine ring.*

5-Aminoimidazole-4-N-succinocarboxamide ribonucleotide $\rightleftharpoons$ 5-Aminoimidazole-4 carboxamide ribonucleotide + Fumarate.

(j) 5-Formamidoimidazole-4-carboxamide ribonucleotide : The last carbon atom required to yield all elements of the purine ring system is now introduced by the formylation of 5-aminoimidazole-4-carboxamide ribonucleotide to yield 5-pormamidoimidazole-4-caroxamide ribonucleotide. The reaction is catalyzed by *transformylase* with N^{10}-formyltetrahydrofolate as formyl donor.

5-Aminoimidazole-4-carboxamide ribonucleotide + N^{10}-formyltetrahydrofolic acid $\xrightarrow{K^+}$ 5-Formamidoimidazole-4-carboxamide ribonucleotide + Tetrahydrofolic acid.

(k) Inosinic acid : Closure of the ring with dehydration by the enzyme *inosinicase* yields inosinic acid (hypoxanthine ribonucleotide).

The synthesis of inosinic acid from elementary precursors can be regarded as the result of the sum of the following artificial composite equations :

(*a*) 2 Glutamine + $2HCOO^-$ + HCO_3^- + Glycine + Aspartate + Ribose 5-phosphate $\longrightarrow$ 2 Glutamate + Inosinic acid + Fumarate + $7H_2O$.

(*b*) 7 ATP + 7 H_2O $\longrightarrow$ 6 ADP + 6 Pi + AMP + PPi + $7H^+$

B. Conversion of Inosinic Acid to Adenine and Guanine Ribonucleotides

Formation of adenylic acid from inosinic acid proceeds via two step reaction. In the first step the formation of adenylosuccinate from inosinic acid is catalysed by the enzyme *adenylosuccinate synthetase* (IMP : L. *asparatate ligase*), with participation of aspartic acid and GTP. In the second step adenylosuccinate is cleaved to yield adenylic acid and fumaric acid. This reaction is catalyzed

by adenylosuccinase (*adenylosuccinate* AMP *lyase*). This is probably the same enzyme which cleaves fumarate from 5-aminoimidazole-4-N-succinocarboxamide ribonucleotide.

Conversion of inosinic acid to guanylic acid also proceeds in a two step process. In the first step inosinic acid is oxidized to xanthylic acid by the enzyme *inosinic acid dehydrogenase* (IMP : NAD *oxidoreductase*) with NAD as the electron acceptor. In the second step xanthylic acid is converted to guanylic acid with ATP and ammonia or glutamine. The reaction is catalyzed by *guanylic acid synthetase* (*xanthosine-5-phosphate :* ammonia ligase). The biochemical transformations converting inosinic acid to adanylic and guanylic acids are indicated in Fig. 25.3.

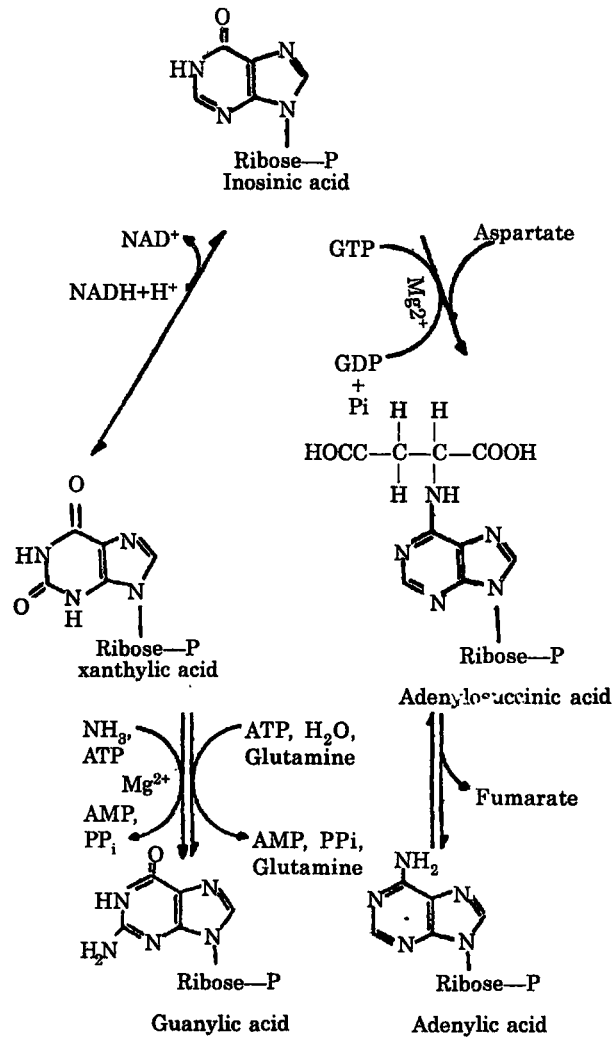


Fig. 25.3. Biosynthesis of adenylic and guanylic acids from inosinic acid.

C. Formation of Purine Nucleotides from Free Bases and Nucleoside

The primary pathways for the synthesis of adenylic and guanylic acids are through the intermediate, *inosinic acid*. However, additional routes for the formation of purine nucleotides are known. These routes may be regarded as '*salvage*' pathways permitting reutilization of purines or purine derivatives derived by catabolism of nucleic acids and nucleotides. It is clear that free bases can be converted to purine nucleotides, since mutants blocked in the *de novo* pathway have been isolated. These mutants are able to grow with one of the four common purine bases, adenine, guanine, hypoxanthine and xanthine. *Nucleotide pyrophosphorylases* catalyze the formation of purine nucleotide from free purine and PRPP. The reversible reactions shown below are catalyzed by three distinct *phosphoribosyl transferases* of *E. coli :* one for IMP and GMP, one for AMP and one for XMP.

$$\text{Hypoxanthine} + \text{PRPP} \rightleftharpoons \text{Inosinic acid} + \text{PPi}$$

$$\text{Guanine} + \text{PRPP} \rightleftharpoons \text{Guanylic acid} + \text{PPi}$$

$$\text{Adenine} + \text{PRPP} \rightleftharpoons \text{Adenylic acid} + \text{PPi}$$

$$\text{Xanthine} + \text{PRPP} \rightleftharpoons \text{Xanthylic acid} + \text{PPi}$$

Conversion of Adenine to Guanine Nucleotides

The adenine nucleus is converted to guanine ribonucleotides by two pathways in *E. coli*. In one pathway, the nitrogen atom at position 1 and the carbon atom at position 2 are lost and then replaced before guanine is formed. The loss of positions 1 and 2 of adenine is because these two positions are incorporated into the imidazole ring in the biosynthesis of histidine. The remainder of the adenine nucleotide, 5-amino-4-imidazolecarboxamide ribonucleotide is an intermediate in purine biosynthesis. In the second pathway the intact adenine ring is converted to guanine through hypoxanthine. The reaction is catalyzed by the action of *adenine deaminase* (*adenine aminohydrolase*).

Conversion of Guanine to Adenine Nucleotides

A distinct pathway for the conversion of guanine to adenine has been discovered. This does not involve reversal of the reactions from IMP to GMP. An enzyme GMP *reductase* (*reduced NAD-GMP-oxidoreductase*) has been isolated from mutants of several species of *Enterobacteriaceae*. The enzyme catalyzes the reductive deamination of GMP to IMP.

$$GMP + NADPH \longrightarrow IMP + NADP + NH_3$$

IMP can then be converted to AMP through the *de novo* purine biosynthetic pathway.

Conversion of Free Purines and Nucleosides to Nucleotides

There are other salvage pathways which convert free purines to nucleosides and nucleosides to nucleotides. A generalised reaction for purine nucleoside formation catalysed by purine *nucleoside phosphonylase* is as follows : Purine + Ribose—1—phosphate ⟶ Nucleoside + Pi

Conversion of nucleosides to nucleotides is catalysed by a *nucleoside kinase*. A generalised reaction is as follows :

$$\text{Nucleoside} + \text{ATP} \xrightarrow[\text{kinase}]{\text{adenosine}} \text{Nucleotide} + \text{ADP}$$

Interconversions of purine bases, nucleosides and nucleotides are shown in Fig. 25.4.

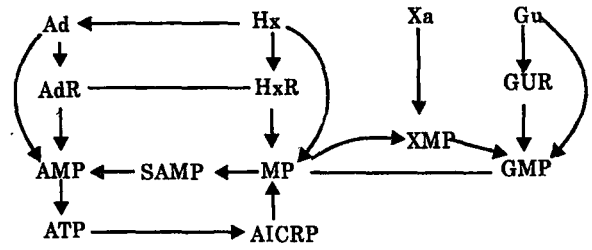


Fig. 25.4. Interconversions of purine bases, nucleosides and nucleotides. The abbreviations used are : A = adenine, AR = adenosine, H = hypoxanthine, HR = inosine, G = guanine, GR = guanosine, Xa = xanthine, AICRP = 5-amino-4-imidazolecarboxamide ribonucleotide, S = succinyl, (Zimmerman and Magasanik, 1964).

25.2 Biosynthesis of Pyrimidine Nucleotides

A major distinction between the metabolic routes leading to the synthesis of purine and pyrimidine nucleotides is the timing in the formation of N-glycosidic bond. This bond is formed in the very early steps in purine synthesis, and the ring system is built upon this foundation. In contrast, the complete pyrimidine nucleus is first synthesized and then attached to ribose-5-phosphate. *Orotic acid*, which contains the pyrimidine nucleus, is the key intermediate introduced in the N-glycosidic linkage. The main enzymatic pathways leading to the formation of orotic acid and conversion to uridylic acid have been elucidated.

A. De Novo Synthesis of Uridylic Acid

In the formation of orotic acid, ammonia is incorporated into the nitrogen atom at position 1 of orotate, carbon dioxide into position 2 and L-asparate into the remainder of the orotate molecule.

(a) Carbamoyl phosphate : The initial step in pyrimidine biosynthesis is the formation of carbamoyl phosphate. Carbamoyl phosphate is synthesized by enzymes utilizing glutamine as the amino group donor. *Carbamoyl phosphate synthetase* isolated from *E. coli* catalyzes a reaction in which two molecules of ATP are consumed per molecule of carbamoyl phosphate synthesized. The enzyme utilizes ammonia as well as glutamine as the amino group donor. However, the affinity for glutamine is much greater than for ammonia.

$$\text{Glutamine} + HCO_3^- + 2ATP + H_2O \rightleftharpoons H_2N{-}\overset{O}{\overset{\|}{C}}{-}OPO_3H_2 + \text{Glutamate} + 2\,ADP + Pi$$

(b) N-carbamoyl-L-asparate : In the second step, the carbamoyl phosphate donates its carbamoyl moiety to the α-amino group of aspartate. This reaction is catalyzed by *asparate transcarbamoylase.* This is the *committed step* in pyrimidine biosynthesis. The enzyme in yeast, *Neurospora* and mammalian cell is subject to end product inhibition by UTP. In *E. coli* CTP is the feedback inhibitor.

$$\text{Carbamoyl phosphate} + \text{L—aspartic acid} \rightleftharpoons \text{N carbamoyl—L—aspartate} + Pi$$

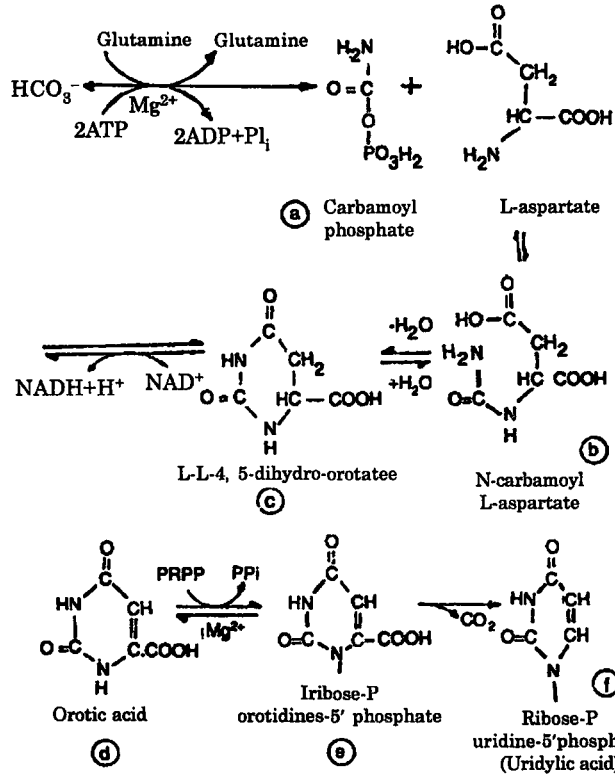


Fig. 25.5. Biosynthesis of uridylic acid.

(c) L-4, 5-Dihydroorotate : N-carbamoyl aspartate is converted to L-4, 5-dihydroorotic acid. This brings about ring closure catalyzed by *dihydroorotase.*

$$\text{N-Carbamoyl aspartate} \underset{+H_2O}{\overset{-H_2O}{\rightleftharpoons}} \text{L-4, 5-dihydroorotate}$$

(d) Orotic acid : L-4, 5-Dihydroorotate is oxidized to orotic acid by the enzyme *dihydroorotate dehydrogenase.* The enzyme is a flavoprotein and transfers the electrons to NAD^+.

$$\text{L-4,5-Dihydroorotata} + NAD^+ \rightleftharpoons \text{Orotic acid} + NADH + H^+$$

(e) Orotidine-5'-phosphate : Orotic acid combines with a phosphoribosyl group from PRPP to form the first nucleotideorotidine-5'-phosphate. The reaction is catalyzed by *orotate phosphoribosyl transferase.* The enzyme is specific for orotic acid and does not react with precursors of orotic acid or related pyrimidines.

$$\text{Orotic acid} + \text{PRPP} \xrightleftharpoons{Mg^{2+}} \text{Orotidine-5'-phosphate} + \text{PPi}$$

(f) Uridylic acid. Orotidine-5'-phosphate is decarboxylated to yield uridylic acid. This irreversible reaction is catalyzed by *orotidine-5'-phosphate decarboxylase.*

$$\text{Orotidine-5'-phosphate} \longrightarrow \text{Uridylic acid} + CO_2$$

The metabolic pathway leading to the synthesis of uridylic acid is given in Fig. 25.5.

B. The Conversion of Uridine-5-Phosphate to UTP and Cytosine Ribonucleotides

The pathway that results in the formation of uridine triphosphate involves consecutive transfer of phosphate from ATP to uridine-5'-phosphate, with intermediate formation of uridine diphosphate.

$$\text{UMP} \xrightarrow[\text{ATP}\ \text{ADP}]{Mg^{2+}} \text{UDP} \xrightarrow[\text{ATP}\ \text{ADP}]{Mg^{2+}} \text{UTP}$$

The *kinases* responsible for catalyzing these reactions are not specific for the base, and will phosphorylate adenine nucleotides.

Uridine nucleotides are intermediates in the conversion of orotic acid into cytosine nucleotides. The only known pathway for the formation of cytosine nucleotides involves amination of UTP.

$$\text{UTP} + \text{Glutamine} + \text{ATP} \xrightleftharpoons{Mg^{2+}} \text{CTP} + \text{Glutamate} + \text{ADP} + \text{Pi}$$

C. Formation of Pyrimidine Nucleotides from Free Bases and Nucleosides

Exogenous uracil, uridine, cytosine and cytidine are readily incorporated into nucleic acids. However, under the usual conditions of growth no thymine and very little thymidine are incorporated.

Hydrolytic deamination of cytosine to uracil and cytidine to uridine have been demonstrated in extracts of *E. coli. Phosphoribosyl transferase* isolated from several species of *lactobacilli and E. coli* catalyzes the formation of UMP from uracil.

$$\text{Uracil} + \text{PRPP} \rightleftharpoons \text{UMP} + \text{PPi}$$

In addition, *nucleoside phosphorylase* catalyzes the formation of uridine from uracil

$$\text{Uracil} + \text{Ribose-1-phosphate} \rightleftharpoons \text{Uridine} + \text{Pi}$$

Uridine is converted to uridylic acid by a *nucleoside kinase.* However, no enzymes have been described for the direct conversion of cytosine or cytidine to cytidylic acid. The pathways for the utilization of pyrimidines and pyrimidine nucleosides are shown in Fig. 25.6.

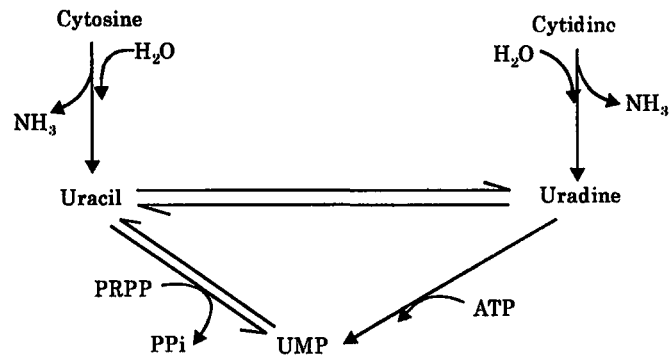


Fig. 25.6. The pathways for the utilization of pyrimidines and pyrimidine nucleosides.

25.3 Biosynthesis of Deoxyribonucleotides

The purine and pyrimidine deoxyribonucleotides of DNA are derived by direct conversion from corresponding ribonucleotides. Thus formation of deoxyribose from ribose occurs at the nucleotide level. Two distinct classes of *ribonucleotide reductase* have been described. One class is represented by the enzyme isolated from *E. coli*, and the other by the enzyme from *L. leichmannii*. Both enzymes catalyze the substitution of the —OH group at position 2′ of ribose by a hydrogen, with retention of configuration at the carbon atom.

Synthesis of deoxyribonucleotides in *E. coli* has been investigated by Reichard and his co-workers. The basic aspects of this system can be summarised as follows :

1. The substrates for reduction are *ribonucleoside diphosphates.*

2. The reduction is catalysed by a *ribonucleoside diphosphate reductase,* which is composed of two non-identical subunits B_1 and B_2 in a 1 : 1 stoichiometry. The schematic structure of the *E. coli* enzyme is given in Fig. 25.7. Protein B_1 has a molecular weight of 160,000, and as isolated, is a dimer of the general structure $\alpha\alpha'$. The two polypeptide chains are of similar size, with identical COOH termini but different-NH_2 termini. B_1 contains binding sites for both ribonucleoside diphosphate substrates and for the nucleoside triphosphates which act as allosteric effectors. B_1 contains two classes of effector binding sites (*h* and I-sites, Fig. 25.7). Allosteric effectors ATP, *d*TTP, and *d*GTP bind to the *h*-site, while ATP and *d*ATP bind to the I-site. B_1 also contains oxidation reduction active sulphydryl groups. Protein B_2 has a molecular weight of 78,000, and consists of two apparently identical polypeptide chains (general structure β_2). Each molecule of B_2 contains two atoms of iron, presumably one per polypeptide chain. Iron is an obligatory cofactor for enzyme activity. Each subunit, B_1 and B_2 by itself is completely inactive. Association of subunits to form the catalytically active enzyme depends upon the presence of 0.01 M Mg^{2+}.

3. The specific hydrogen donor for the reduction is *thioredoxin* in the sulphydryl form. Thioredoxin is a small protein (108 amino acids MW = 12,000 in *E. coli*), containing an oxidation-reduction active disulphide with the sequence (-Cys-Gly-Pro-CYs). This sequence represents the active centre of thioredoxin isolated from all sources.

4. The oxidized form of thioredoxin is reduced in the cell by a specific FAD-protein, *thioredoxin reductase,* with NADPH as the hydrogen donor. *Thioredoxin reductase* from *E. coli* contains two moles of FAD, and has a molecular weight of 66,000, with the general structure $(FAD)_2$ α_2. Each polypeptide chain contains one oxidation-reduction active disulphide (-Cys-Ala-Thr-Cys). The reaction mechanism involves a stepwise reduction of FAD and oxidation-reduction disulphides. Since oxidation-reduction active disulphides also appear in *ribonucleotide-reductase* the overall sequence of electron transfer from NADPH to

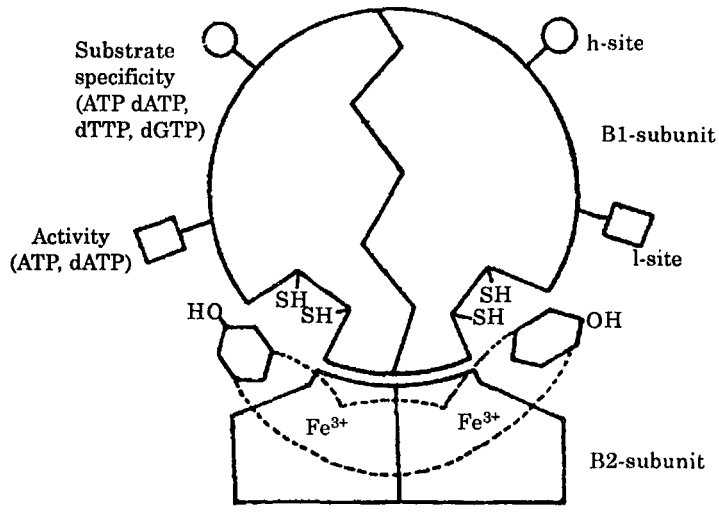


Fig. 25.7. Schematic structure ribonucleoside diphosphate reductase of *E. coli.*

ribonucleotides involves a shuttle of $S_2/(SH)_2$ interchanges as described in Fig. 25.8.

5. All four nucleoside diphosphates, ADP, GDP, CDP and UDP, are converted to the corresponding deoxyderivatives by the same *reductase* system.

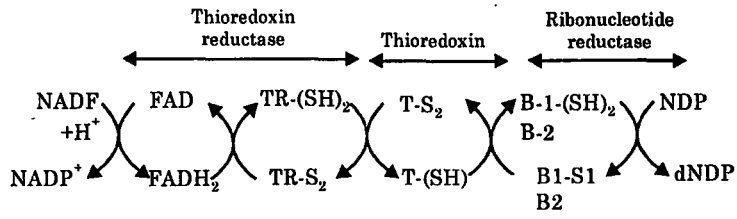


Fig. 25.8. Involvement of oxidation-reduction active disulphides in ribonucleotide reduction in *E. coli.*

6. The substrate specificity of *ribonucleotide reductase* is controlled by allosteric mechanisms. Nucleoside triphosphates either act as stimulators or inhibitors and thus regulate the *reductase* system. The effector binding studies to the expression of enzyme activity has revealed the existence of two classes of sites (*h* and I) on b_1 (Fig. 25.7). The binding of effectors (ATP or dATP) to I-sites (= activity sites) regulates the general level of activity, with dADTP acting as a negative and ATP as a positive effector. The binding of effectors to *h*-sites (= specificity sites) results in conformational changes at the active sites. This induces preferential binding of one or the other substrate. The multiplicity of effectors and effector binding sites make possible a large number of different states of the enzyme with different specificity. The allosteric regulation of *ribonucleotide reductase* from *E. coli* is presented in Table 25.1.

Table 25.1

ALLOSTERIC REGULATION OF RIBONUCLEOTIDE REDUCTASE FROM E. COLI

Effector binding to		*Reduction of*			
I-sites	*h-sites*	*CDP*	*UDP*	*GDP*	*ADP*
0	ATP	+	+	0	0
0	dTTP	+	+	+	+
0	dGTP	0	0	+	+
ATP	ATP or dATP	+	+	0	0
ATP	dTTP	—	—	+	(+)
ATP	dGTP	nd	nd	(+)	(+)
dATP	Any effector	—	—	—	—

The schematic interpretation of the major allosteric effects on deoxyribonucleotide synthesis is presented in Fig. 25.9. These results refer only to the purified enzyme. The existence of the implied control mechanisms in living cells have yet to be demonstrated.

Ribonucleotide reductase in L. leichmanii differs from that in *E. coli*. The major aspects of this system can be summarised as follows :

1. The substrates for reduction are ribonucleoside triphosphates, ATP, GTP, CTP and UTP.

2. The reduction is catalyzed by a *ribonucleoside triphosphate reductase,* which is a monomer with a molecular weight of 76,000.

3. The activity of the enzyme is completely dependent on the presence of 5'-deoxy-5'-adenosyl-cobalamin (Vitamin B_{12} coenzyme).

4. Vitamin B_{12} coenzyme helps in the transfer of hydrogen from the *thioredoxin-thioredoxin reductase* system to the reduction of all four ribonucleoside triphosphates.

5. The allosteric regulation of the *L. leichmannii reductase* activity by various nucleoside triphosphates appears related, but is distinct from that for the *E. coli* enzyme. The most strong

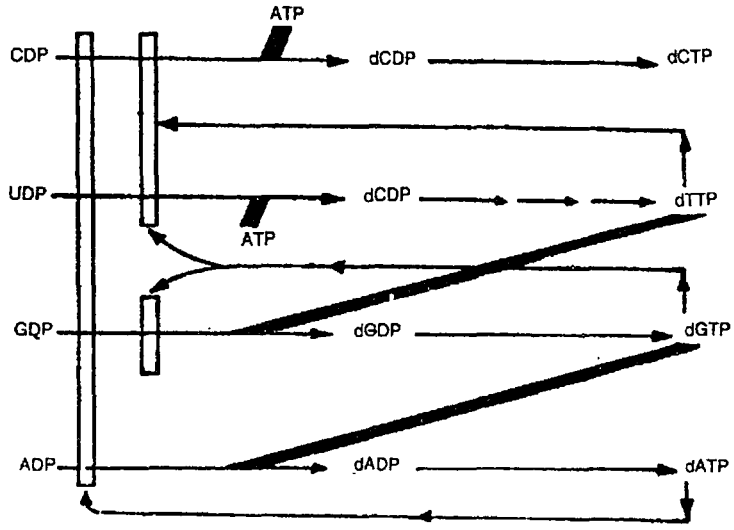


Fig. 25.9. Schematic interpretation of major allosteric effect on deoxyribonucleotide synthesis. The open bars stand for negative effects, the closed bars for positive effects.

positive effectors are dATP for the reduction, of CTP, dCTP for UTP reduction, dTTP for GTP reduction, and dGTP for ATP reduction. No strong negative effector has been found for the *L. leichmannii enzyme.*

25.4 Regulation of Nucleotide Synthesis

The formation of nucleotides for the biosynthesis of nucleic acids is the result of a long and complex chain of enzyme reactions. The synthesis of both purine and pyrimidine nucleotides is under metabolic control, which involves both *feedback inhibition* and *repression by end products.*

Regulation of the synthesis of the purine nucleotides has been summarized by Magasanik. The principal facts are as follows :

1. The *committed step* in the purine biosynthetic pathway is the formation of 5-phosphoribosyl-1-amine, catalyzed by *glutamine phosphoribosyl pyrophosphate amidotransferase.* This reaction is inhibited by the mono-, di-, and triphosphates of inosine, adenosine : and guanosine. Thus the end product of the pathway shuts the entire pathway at the very beginning.
2. GMP inhibits *IMP dehydrogenase,* which converts IMP to XMP on the route to GMP.
3. AMP and ADP inhibit adenylosuccinate synthesis, which converts IMP to adenylosuccinate.
4. ATP inhibits *GMP reductase,* which catalyses the conversion of GMP to IMP.
5. AMP inhibits *adenine phosphoribosyl transferase,* which converts adenine to AMP.
6. ADP inhibits *hypoxanthine phosphoribosyl transferase,* which converts hypoxanthine to AMP.
7. GTP inhibits both *guanine and xanthine phosphoribosyl transferases,* which convert guanine and xanthine to GMP and XMP, respectively.
8. ATP activates *AMP deaminase* which converts AMP to IMP. These controls prevent the formation of adenine and guanine nucleotides when either is present in excessive amounts within the cell. Since adenine and guanine nucleotides are interconvertible, there is no requirement for *de novo* synthesis of the purines. Some of the regulatory steps in purine nucleotide synthesis are shown in Fig. 25.10.

Biosynthesis of pyrimidine nucleotides is regulated at various stages in the metabolic pathway. The *committed step* in the pyrimidine biosynthetic pathway is the formation of N-carbamoyl aspartate, catalyzed by *aspartate transcarbamoylase.* The enzyme isolated from *Neurospora,* yeast, and mammalian cells is subject to end-product feedback inhibition by UTP, while in *E. coli* CTP is the feedback inhibitor. The enzyme isolated from *E. coli* is highly regulated.

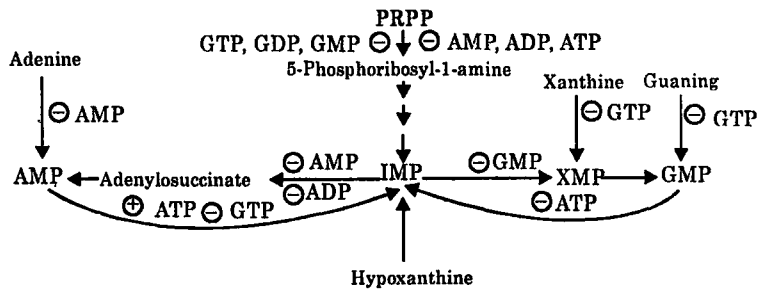


Fig. 25.10. Regulation of synthesis of purine nucleotides. (+) indicates activation, (–) indicates inhibiton.

The enzyme *aspartate transcarbomoylase* from *E. coli* has a molecular weight of 300,000. It dissociates on treatment with mercurials into two subunits. One type of subunit possesses the entire catalytic activity, and is insensitive to CTP. This *catalytic subunit is* made up of two trimers (MW = 100,000 for each trimer). Thus, there are six polypeptides (MW = 33,500 for each polypeptide) per molecule of native enzyme. The second type of subunit consists of three dimers and is known as the *regulatory subunit.* Again there are six polypeptides (MW = 17,000 for each polypeptide) of regulatory subunits per molecule of native enzyme which can bind sin molecules of CTP. Of the total enzyme protein, the catalytic subunits represent 67% and the regulatory subunits and 33% of the structure. Six atoms of Zn are present in the native enzyme.

The catalytic subunits of the enzyme interact with aspartate, and the regulatory subunits interact with CTP or other inhibitors and activators. The interaction at the regulatory subunits prevents interaction at the catalytic subunits. Regulation of enzyme activity exhibits an interesting variation in species. The enzyme from *E. coli, A. aerogenes,* and S. *marcescens* are all subject to feedback inhibition by CTP, that from *P. fluorescens* is most strongly inhibited by UTP, and that from lettuce seedlings is most sensitive to UMP. Furthermore, the enzyme from *B. subtilis* seems not to be subject to feedback inhibition at all, but is subject to end product repression.

ATP is the immediate source of energy for the synthesis of nucleotides because PRPP is the starting point for the formation of all nucleotides, and large amounts of ATP are required for the formation of this key compound. The enzyme *kinase,* which catalyzes the synthesis of PRPP, is strongly inhibited by ADP and GDP. Thus when the energy supply (ATP) is low, the synthesis of PRPP is inhibited.

25.5 Catabolism of Nucleotides

Nucleic acids within the cells are hydrolyzed by a variety of enzymes, and are eventually degraded to mononucleotides, nucleopyramidine bases.

A. Purine Catabolism

The breakdown of purine nucleotides has been studied extensively. Adenine and guanine nucleotides are converted to adenosine and guanosine, respectively, by the enzyme *phosphatase. Nucleosidase* converts adenosine and guanosine to adenine and guanine, respectively. Alternatively, adenine and guanine and their nucleosides and nucleotides can be deaminated hydrolytically under the influence of appropriate *deaminases.* These reactions lead to the production of hypoxanthine and xanthine from adenine and guanine, respectively.

Hypoxanthine and xanthine are oxidized to *uric acid.* The reaction is catalyzed by the related enzymes, *xanthine oxidase or xanthine dehydrogenase.* The terminology reflects the nature of the ultimate electron acceptor; oxygen in case of milk *xanthine oxidase,* ferredoxin for the *dehydrogenase* from *Micrococcus lactilyticus,* and NAD for the chicken liver *dehydrogenase.*

Certain microorganisms are capable of utilizing purines as their major carbon and nitrogen sources. Two degradative pathways have been worked out in some detail. An *oxidative pathway* in certain *Pseudomonas* and fungi is initiated by the action of *uricase.* The pathway proceeds through allontoin, allonotoic acid, ureidoglycolate to urea and glyoxalate (Fig. 25.11). A *fermentative pathway* of purine degradation exists in *Clostridium acidiurici* and Cl. *cylendrosporum.* The series of hydrolytic reactions starting from xanthine is outlined in Fig. 25.12. The path of this sequence resembles the reverse of purine biosynthesis, except that non-phosphorylated derivatives are involved.

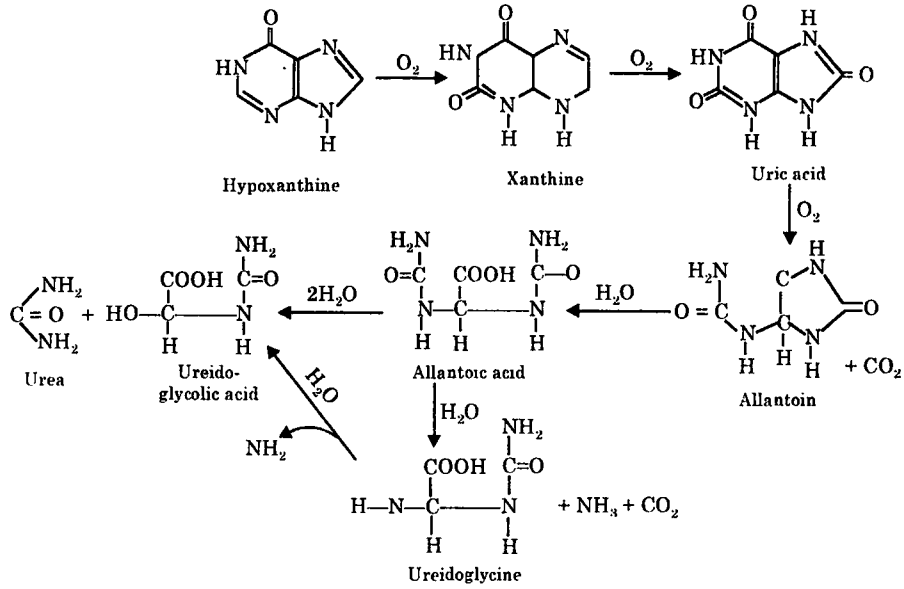


Fig. 25.11. Oxidative pathway of purine degradation.

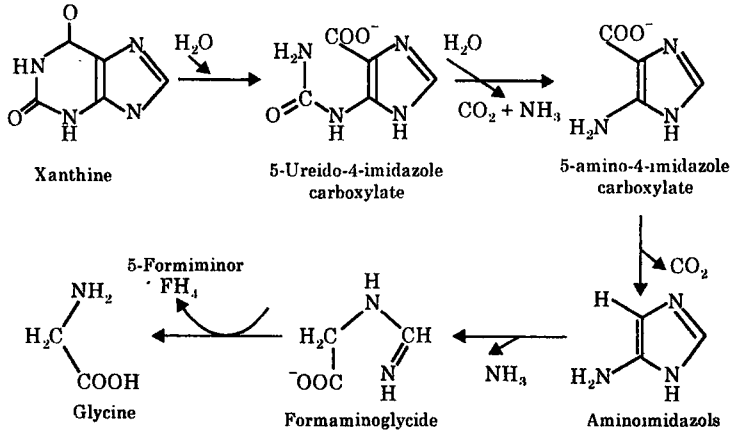


Fig. 25.12. Fermentative pathway of purine degradation.

B. Pyrimidine Catabolism

The catabolism of pyrimidine nucleotides, like that of purine nucleotides, involves *dephosphorylation, deamination,* and *cleavage of glycosidic bonds.*

Removal of the amino group of cytosine derivatives to form the corresponding uracil compounds is necessary before the degradation of the pyrimidine ring system. Cytosine is deaminated to uracil by *cytosine deaminase*. This reaction has been demonstrated in yeast and other microorganisms. Cytidine is deaminated to uridine by *cytidine deaminase*. This enzyme is widespread in animal tissues as well as in bacteria.

The catabolic pathways for uracil and thymine involves reduction of pyrimidines to the dihydro derivatives. The latter substances are hydrolyzed, with the ring opening to the corresponding ureidoderivatives. These are further hydrolyzed to produce CO_2, ammonia and β-alanine or its methylated derivative (Fig. 25.13).

An alternative route of pyrimidine catabolism is observed in certain bacteria, particularly species of *Corynebacterium* and *Mycobacterium*. The pathway proceeds by initial oxidation of uracil and thymine at carbon 6 to yield *barbituric acid* and 5-*methyl barbituric* acid, respectively. This reaction is catalyzed by a single enzyme *uracil-thymine oxidase*. An enzyme, *barbiturase* catalyzes the hydrolysis of barbiturates to *urea* and *malonate or* methyl *malonate* (Fig. 25.14).

25.6 Formation of Coenzyme Nucleotides

All the ribose-containing nucleotides found in RNA also play other important metabolic roles. Biosyntheses of some coenzymes which contain nucleotides and their derivatives are described in the following paragraphs.

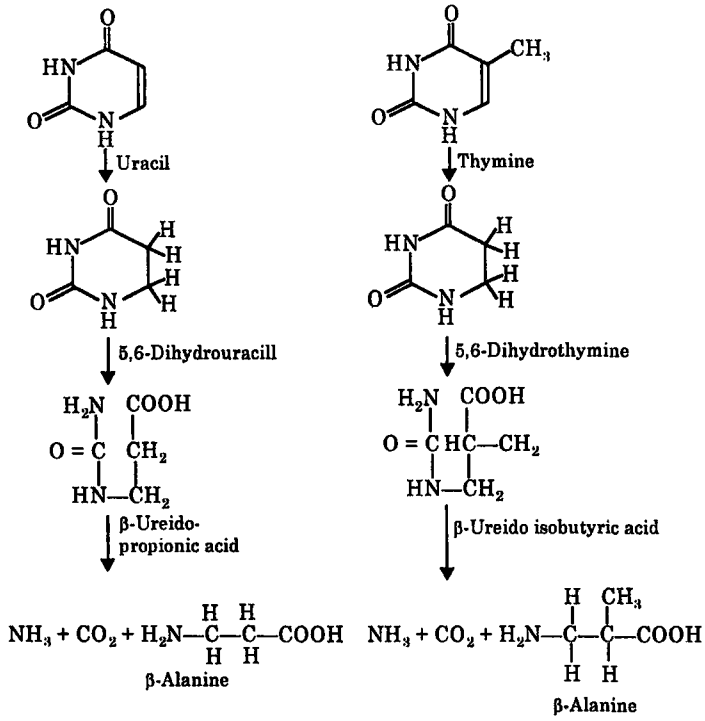


Fig. 25.13. Catabolic pathways of uracil and thymine.

A. Flavin Nucleotides

Riboflavin, in its free form, is a relatively rare natural product which occurs in significant amounts only in the culture media of some microorganisms and in the retina, urine, milk and seminal fluid. Riboflavin occurs in the combined form as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FMN and FAD act as the prosthetic groups of a number of enzymes. The structures of riboflavin, FMN and FAD are given in Fig. 25.15.

The pathway of riboflavin synthesis from the gaunosine derivative (I) has been studied in various moulds, yeasts, and bacteria and is shown in Fig. 25.16. The state of the phosphorylation of guanosine derivative (RI) is uncertain. The first step is the loss of C-8 of the purine ring by hydrolytic removal

of formate. This leads to the opening of the ring, with the formation of 6-hydroxy-2, 4, 5-triamino-pyrimidine (II). Reduction of II yields the corresponding ribityl (R) derivative (III). Replacement of an amino group by a keto group yields the flavin precursor, 4-ribitylamino-5-amino-2, 6-dihydroxy-pyrimidine (IV). Additional carbon atoms to build the benzene ring of riboflavin are supplied in two stages. In the first stage *acetoin* (CH_3—CO—CHOH—CH_3) or *diacetyl* (CH_3—CO—CO—CH_3) combines with diamino precursor to yield 6, 7-dimethyl-8-ribityllumazine (V). Completion of the riboflavin ring (VI) requires additional four carbon atoms, which are supplied by a second unit of diacetyl. This is not done directly, but by the transfer of the diacetyl unit forms a second molecule of 6, 7-dimethyl-8-ribityllumazine. The reaction is catalyzed by *riboflavin synthetase,* which has binding sites for two molecules of lumazine. This is a remarkable reaction, discovered by Plaut, wherein the diazine ring of one molecule is ruptured and a four-carbon fragment is added to the second molecule of lumazine, as shown by the distribution of 14C (indicated by the asterisks). The diamino precursor (IV) is regenerated in this process. Flavin mononucleotide (FMN) is formed from riboflavin and ATP in a reaction catalyzed by *flavokinase.*

$$\text{Riboflavin} + \text{ATP} \xrightarrow{Mg^{2+}} \text{FMN} + \text{ADP}$$

Flavin adenine dinucleotide (FAD) is formed from FMN by a reversible reaction catalyzed by *flavin nucleotide pyrophosphorylase*

$$\text{FMN} + \text{ATP} \underset{Mg^{2+}}{\rightleftharpoons} \text{FAD} + \text{PPi}$$

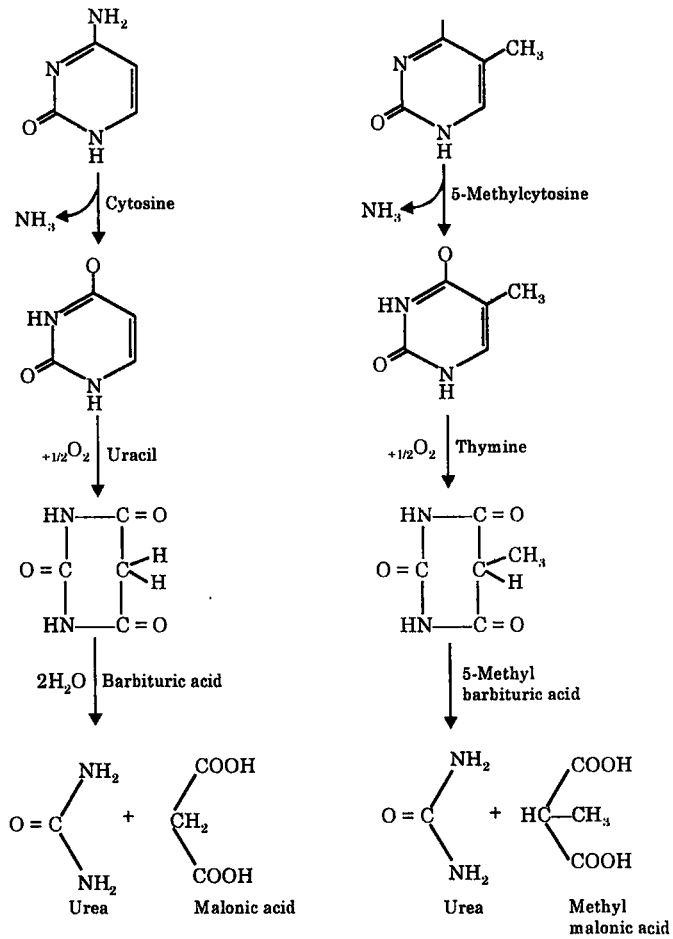
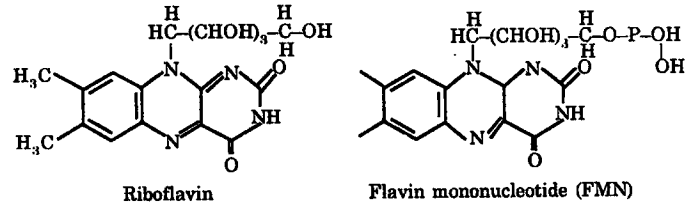
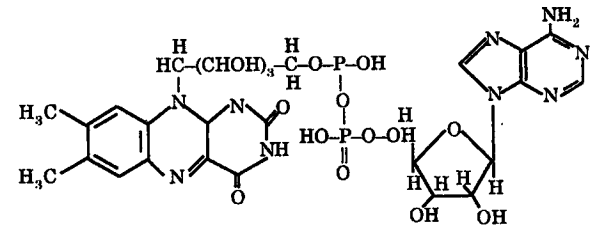


Fig. 25.14. Pathway of bacterial metabolism of pyrimidine.



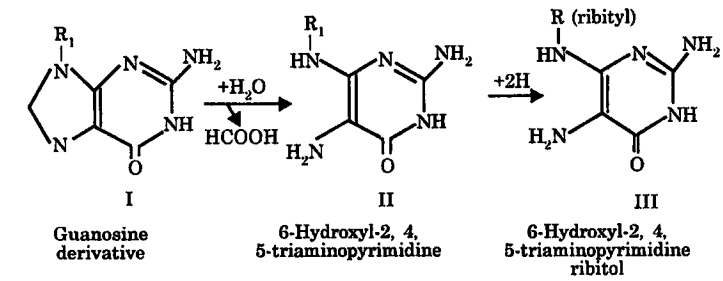
Riboflavin

Flavin mononucleotide (FMN)



Flavin adenine dinucleotide (FAD)

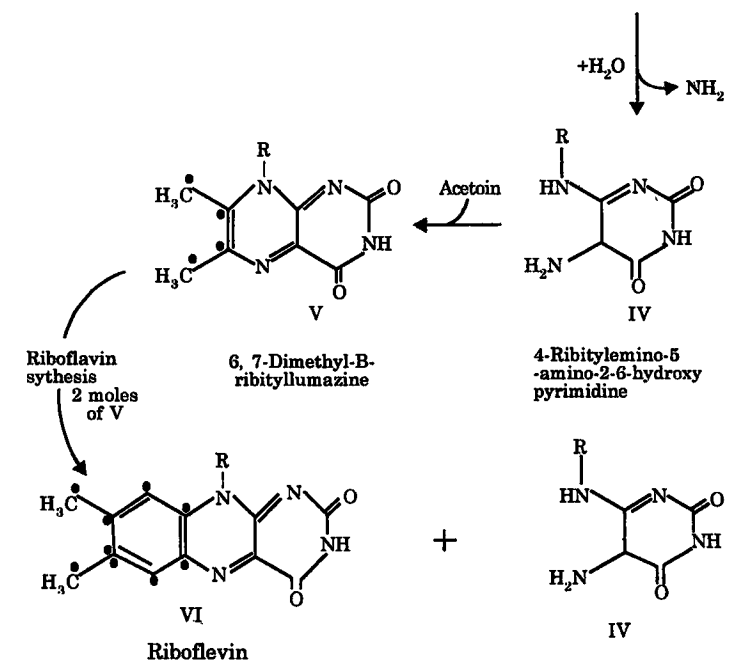
Fig. 25.15. Structures of riboflavin, FMN and FAD.



I — Guanosine derivative

II — 6-Hydroxyl-2, 4, 5-triaminopyrimidine

III — 6-Hydroxyl-2, 4, 5-triaminopyrimidine ribitol



IV — 4-Ribitylemino-5-amino-2-6-hydroxy pyrimidine

V — 6, 7-Dimethyl-B-ribityllumazine

VI — Riboflevin

Riboflavin sythesis 2 moles of V

Acetoin

Fig. 25.16. Postulated pathway for riboflavin biosynthesis.

B. Folic Acid (Peteroylglutamic Acid)

Folic acid also originates from a derivative of guanosine. GTP (I) is directly utilized for pteridine synthesis as shown in Fig. 25.17. The first step is the loss of C-8 of the purine ring by the hydrolytic removal of formate. This leads to the opening of the ring. This is followed by a simple ring closure between the carbonyl and adjacent amino group to form dihydroneopterin triphosphate (IV), through the postulated intermediates (II) and (III). A single protein form *E. coli* (MW = 300,000) catalyzes both reactions. Dihydroneopterin triphosphate is completely dephosphorylated, and is then converted to 2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine (V) with the removal of the

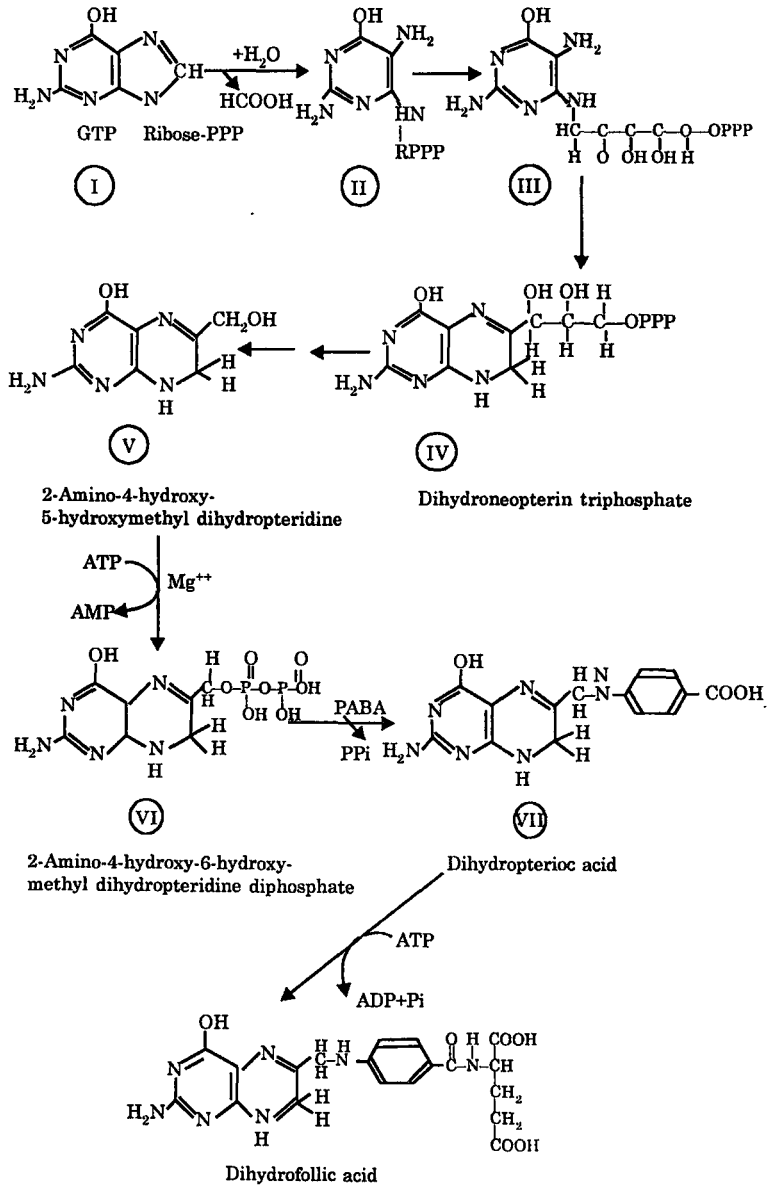


Fig. 25.17. Postulated pathway for folic acid biosynthesis.

two-carbon compound, glycoldehyde. The pyrophosphoric ester (VI) of compound (V) reacts with *p*-amino benzoic acid to yield dihydropteroic acid (VIII). Compound (VII) reacts with glutamic acid to yield dihydrofolic acid.

C. Vitamin B_{12} Coenzyme

The 5, 6-dimethylbenzimidazole part of the vitamin B_{12} coenzyme may be derived from 6, 7-dimethyl-8-ribityllumazine in a process resembling that of riboflavin synthesis. But in this process the riboflavin formed is hydrolytically degraded to remove the pyrimidine ring and form the imidazole ring. The C-2 atom of 5, 6-dimethylbenzimidazole is derived from the carbon-1′ of the ribityl portion of riboflavin. It is not known whether riboflavin is an obligatory intermediate in the biosynthesis of the 5, 6-dimethyl-benzimidazole or whether both are formed *via* branching pathways from the common 6, 7-dimethyl-8-ribityllumazine precursor (Fig. 25.18).

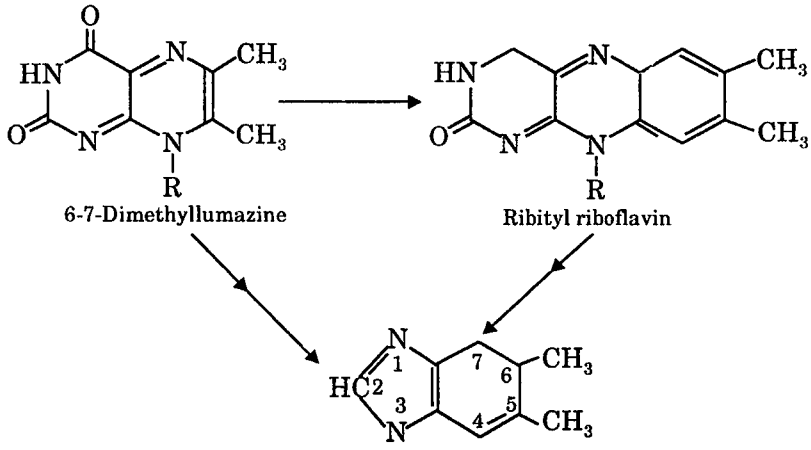


Fig. 25.18. Biosynthesis of 5, 6-dimethylbenzimidazole part of vitamin B_{12}.

D. Pyridine Nucleotides

Nicotinamide adenine dinucleotide (NAD) consists of a molecule of nicotinamide mononucleotide and one of adenine mononucleotide. The union between these two molecules is formed by condensation between their respective phosphate groups. An enzyme NAD *pyrophosphorylase* catalyzes the reaction between nicotinamide mononucleotide and ATP, which donates the adenine mononucleotide component.

Nicotinamide mononucleotide + ATP ⟶ NAD + PPi

NAD can be converted into nicotinamide adenine dinucleotide phosphate (NADP) by simple phosphate transfer from a further molecule of ATP :

$$NAD + ATP \xrightarrow{Mg^{2+}} NADP + ADP$$

The third phosphate group present in NADP is attached to the 2′ of the ribose ring of the adenosine moiety (Fig. 25.19).

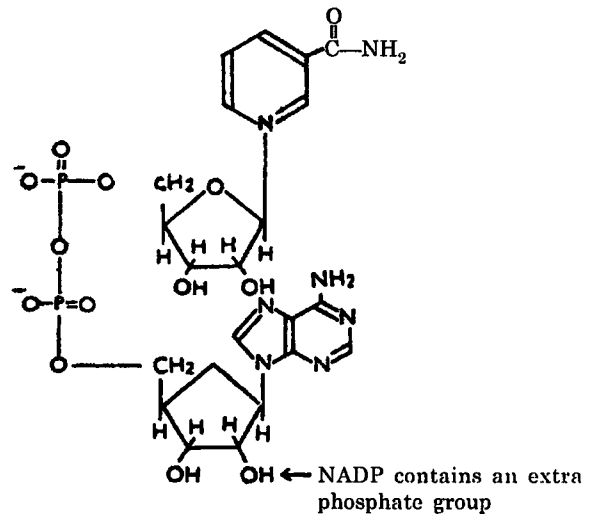


Fig. 25.19. Structure of nicotinamide adenine dinucleotide (NAD).

E. Coenzyme A (CoA)

The complete structure of coenzyme A is shown, in Fig. 25.20. Coenzyme A is composed of adenosine-3′-phosphate-5′-pyrophosphate bound in ester linkage to the vitamin pantothenic acid, which in turn is attached to amide linkage to β-mercaptoethylamine. Three molecules of ATP are required in the synthesis of coenzyme A. One molecule of ATP is required to form 4′-phosphopantothenic acid from pantothenic acid. The 4′-phosphopantothenic acid reacts with cysteine to form 4′-phosphopantothenylcystein, and the latter is decarboxylated to yield 4′-phosphopantothenylcystein, and the latter is decarboxylated to yield 4′-phosphopantetheine. The sequence of reactions are as follows :

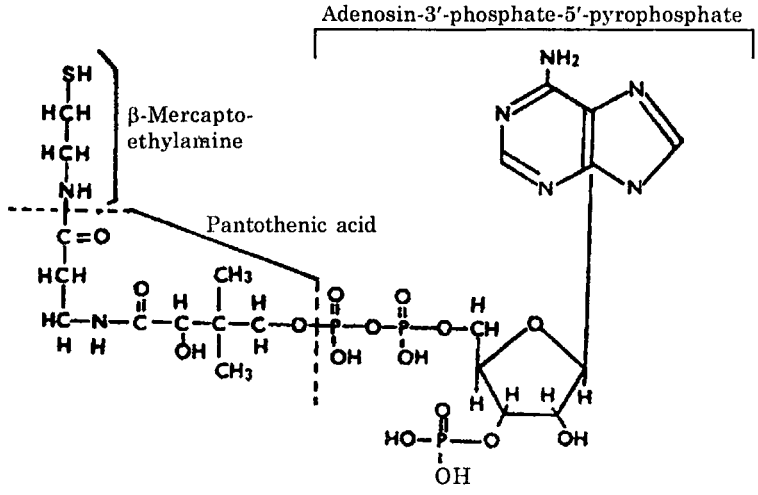
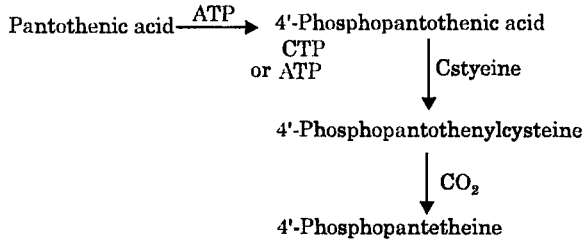
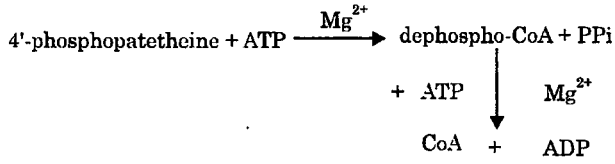


Fig. 25.20. Structure of coenzyme A.



4′-Phosphospantetheine reacts with another molecule of ATP to form dephospho-CoA. In this reaction the adenosine monophosphate group becomes attached through its phosphate group to that of the 4′-phosphopantetheine. The third molecule of ATP is required to introduce a phosphate group in the 3′ position of the ribose ring. This converts dephospho-CoA into CoA. The sequence of reactions is as follows :

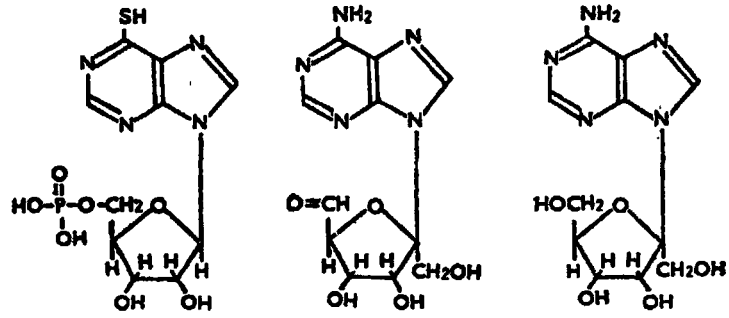


25.7 Inhibitors of Nucleotide Synthesis

The bacterial cells and tumour cells divide much more frequently than the normal somatic cells of adult tissues. The former types, therefore, have high requirements of nucleotides for nucleic acid synthesis. The inhibitors, which block the synthesis of nucleotides, prevent the growth of bacterial and tumour cells more drastically than the host organism. His partial selective toxicity of such inhibitors is the basis for their use in chemotherapy. Most chemical inhibitors are structural analogues of compounds normally required for the synthesis of nucleotides. The structural analogues are powerful competitive inhibitors of various enzyme-catalyzed reactions of nucleotide synthesis. Inhibition of nucleotide synthesis by compounds related to purines, pyrimidines, glutamine, aspartic acid and folic acid are described in the following paragraphs.

A. Purine Analogues

The purine analogue 6-mercaptopurine (Fig. 25.21) is converted to the ribonucleotide. This ribonucleotide is a potent inhibitor of PRPP *transferases* which convert purines into corresponding ribonucleotides. In addition, 6-mercaptopurine ribonucleotide is a potent inhibitor of both steps in the conversion of inosinic acid to adenytic acid, and conversion of inosinic to xaynthelic acid, a precursor of guanylic acid. This results in the inhibition of nucleic acid synthesis. This inhibitor is being used as an antitumour agent in the treatment of certain types of cancer.



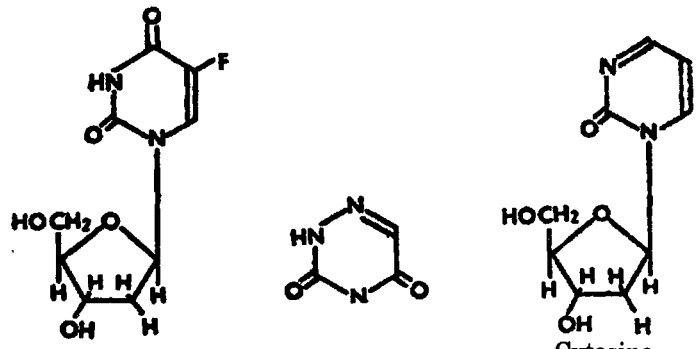
6-Mercaptopurine ribonucleotide — Decoyinine — 6-Amino-9-D-psicofuranosyl purine

Fig. 25.21. Structures of some purine analogues.

The antibiotics psicofuranine and decoyinine isolated from *Streptomyces hygroscopicus var, decoyicus* have a nucleoside type structure (Fig. 25.22). The antibiotics inhibit nucleic acid synthesis in bacteria by blocking the final stage in the bisynthesis of GMP, *i.e.,* the amination of XMP.

B. Pyrimidine Analogues

Some halogenated pyrimidines, particularly 5-fluorodeoxyuridine, are potent inhibitors of DNA synthesis. 5-Fluorodeoxyuridine inhibits the *thymidylate synthetase* reaction. In contrast, the corresponding chloro, brormo, and iodo derivatives do not inhibit DNA synthesis. These derivatives are readily incorporated into DNA, replacing thymine and yielding defective DNA molecules. The van der Walls' replacing thymine and yielding defective DNA molecules. The van der Waals' radii of the chloro, bromo, and iodo groups are not too different from that of methyl, the 5-substituent in thymine. The fluoro-group, on the other hand, is very small and more nearly resembles a hydrogen, as in uracil, than the methyl of thymine. The fluorinated derivative (Fig. 25.22), therefore, competes, with uracil derivatives in the *thymidylate synthetase* reaction, while the chlorobromo- and iodo-derivatives act as thymine analogues in the assembly of DNA.



5-Fluorodeoxyuridinel — 6-Azauracil — Cytosine arabinoside

Fig. 25.22. Structures of some pyrimidine analogues.

An additional pyrimidine derivative, 6-azauracil, (Fig. 25.22), inhibits the synthesis of uridylic acid. The compound inhibits the decarboxylation of orotidylic acid to uridylic acid.

A nucleotide of natural origin (but not derived from nucleic acids), *cytosine arabinoside,* isolated from sponges, has been found to inhibit nucleic acid synthesis. This compound is a potent antileukemic agent.

C. Glutamine Analogues

The antibiotics, azaserine (O'-diazoacetyl-L-serine) and DON (6-diazo-5-oxo-L-norleucine) (Fig. 25.23), isolated from *Streptomyces* are structurally related to glutamine. These compounds, therefore, inhibit to varying extents, those aminations in which glutamine serves as the amino group donor. Two glutamine-dependent aminations, one involves in the conversion of PRPP to phosphoribosyl-amine and the other in the conversion of formylglycinamide ribonucleotide to the corresponding amidine in purine biosynthesis, are strongly inhibited by these glutamine analogues.

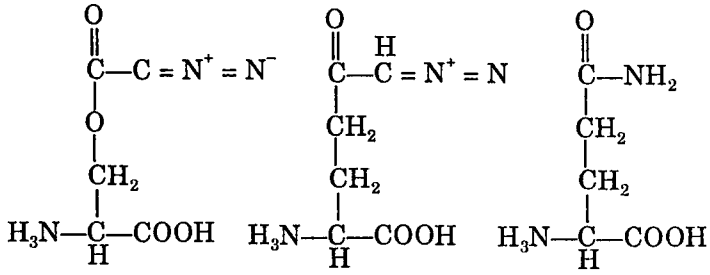


Fig. 25.23. Structures of glutamine analogues.

D. Aspartic Acid Analogue

An antibiotic hadacidin (N-formyl hydroxy amino acetic acid) (Fig. 25.24), isolated from *Penicillium spp.* is structurally related to aspartic acid. The compound inhibits the amination reaction in which aspartic acid serves as amino group donor. The enzyme *adeynlosuccinate synthetase* converts IMP to AMP in a two-step reaction with aspartic acid as the amino group donor. Hadacidin inhibits this reaction and blocks the synthesis of AMP. The antibiotic has little or no antagonistic activity against L-aspartate in other reactions involving this amino acid. For example, the conversion of 5-amino-4-imidazole-N-succinocarboxylic acid ribonucleotide to the corresponding amide, which also requires L-aspartate, is not affected by hadacidin. Again, hadacidin only very weakly inhibits the activity of L-aspartate in pyrimidine biosynthesis. Apparently hadacidin significantly hinders the metabolic activity of L-aspartate only under the specific conditions applying to the amidation of IMP.

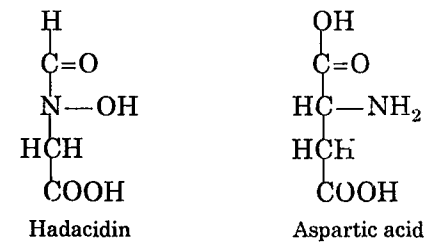


Fig. 25.24. Structure of aspartic acid analogue.

E. Mycophenolic Acid

The antibiotic, mycophenolic acid (Fig. 25.25) isolated from *Penicillium stoliniferum* inhibits IMP-*dehydrogenase* (IMP-NAD *oxidoreductase*) which converts IMP to XMP. The enzyme preparations from fungi and mammalian cells are highly sensitive to the antibiotic, but that from *E. coli* is insensitive. This contrasting effect of the antibiotic on IMP *dehydrogenases* indicates significant structural differences in the enzymes from prokaryotic and eukaryotic cells.

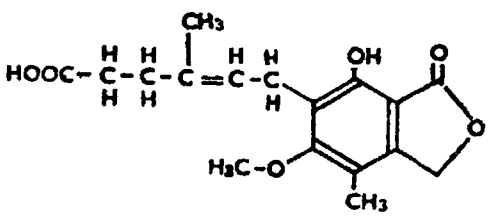


Fig. 25.25. Structure of mycophenolic acid.

F. Folic Acid Analogues

Folic acid derivatives act as coenzymes in one-carbon transfer reactions in the biosynthesis of purines and pyrimidines. A number of compounds which interfere with the synthesis of folic acid or its derivatives have been found to possess antibacterial or antitumour activity.

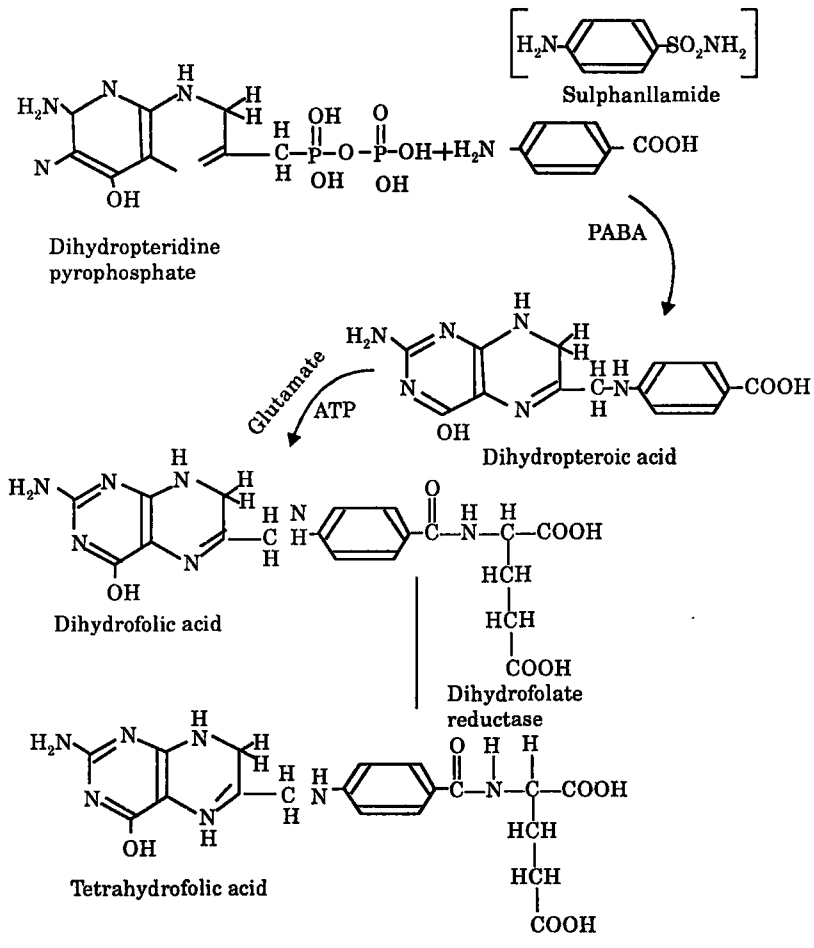


Fig. 25.26. The final stages of folic acid biosynthesis.

The antibacterial activity of sulphonamides is due to their structural similarities with para-amino-aminobenzoic acid (PABA). Some bacteria have a requirement of PABA as a growth factor for synthesis of folic acid. The biosynthesis of folic acid proceeds to the dihydropteridine pyrophosphate derivative. This derivative then reacts with PABA with loss of pyrophosphate group to form dihydropteroic acid (Fig. 25.26). Sulphanilamide and other sulphonamides inhibit this reaction in an apparently competitive manner. The effectiveness of sulphonamides in combating bacterial infections in man probably depends on the fact that man requires folic acid and cannot synthesize it from PABA. Thus, sulphonamides inhibit a metabolic reaction essential for certain bacteria without affecting the metabolism of host who does not make folic acid from PABA.

The antagonists of folic acid, *e.g.*, aminopterin and amethopterin (methotrexate) are some of the most effective antitumour agents. The other antagonists are pyrimethamine and proguanit, which are used for the treatment of malaria. These analogues of folic acid inhibit *dihydrofolate reductase*, which converts dihydrofolic acid to tetrahydrofolic acid. Although most living cells depend upon the enzyme *dihydrofolate reductase*, the enzyme evidently differs in structure from one organism to another. The antagonists, therefore, show specificity in their inhibitory action. Fig. 25.27 gives the structure of some antagonists of folic acid which inhibit dihydrofolate reductase.

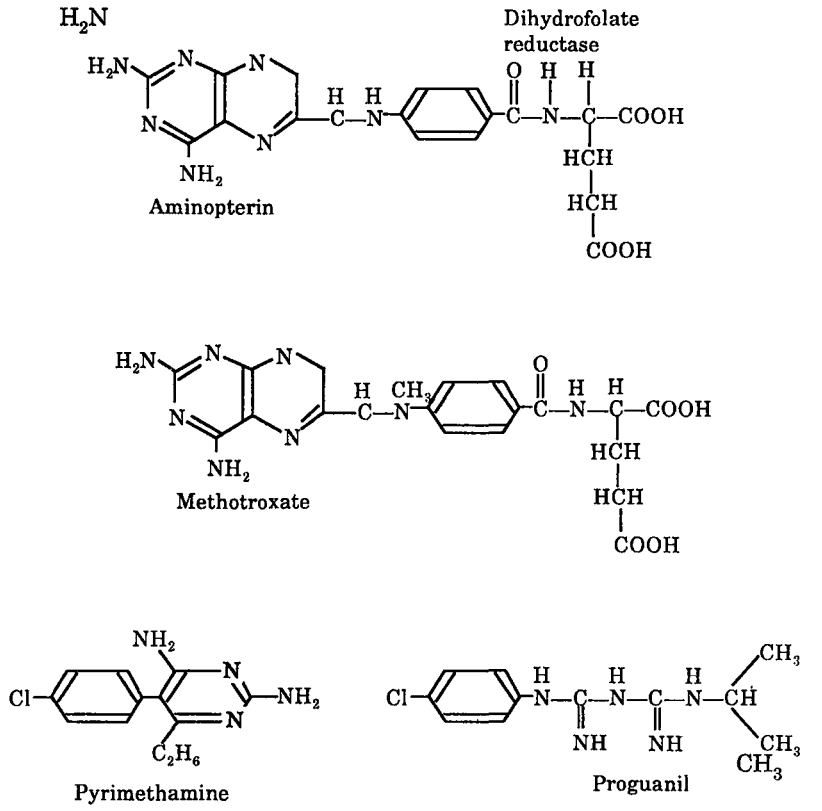


Fig. 25.27. Structures of some analogues folic acid.

25.8 Replication of DNA

One of the most important properties of DNA is that it can make exact copies of itself. This process is called *replication,* and is the very basis of life. The two strands of a DNA double helix are united by hydrogen bonds between the purine and pyrimidine base pairs. When the hydrogen bonds break, the two strands separate and unwind (Fig. 25.28). The nucleus contains free nucleotides which form the *nucleotide pool.* The nucleotides include those containing *adenine, guanine, cytosine* and *thymine* nitrogenous bases.

These free nucleotides pair with the nucleotides of the two separated strands by means of hydrogen bonds. Free *adenine* nucleotide pairs with the *thymine* nucleotide of the strand, and fee *guanine* nucleotide with the *cytosine* nucleotide of the strand, etc. (A-T and G-C pairing). In this way a new strand is formed around each old strand. The result of replication is the formation of two double helices, each identical to the original double helix.

DNA is found mostly in the chromosomes. When the chromosome divides into two during mitosis (actually during interphase) the daughter chromosomes have identical DNA double helices. Now, all the cells in the body are ultimately derived from the zygote by repeated division. It thus follows that they all have exactly similar DNA. Replication ensures that the genes, which are segments of the DNA molecule, are present in identical sets in all cells of the body of an individual. DNA fulfils the requirement of a genetical material : the ability to replicate.

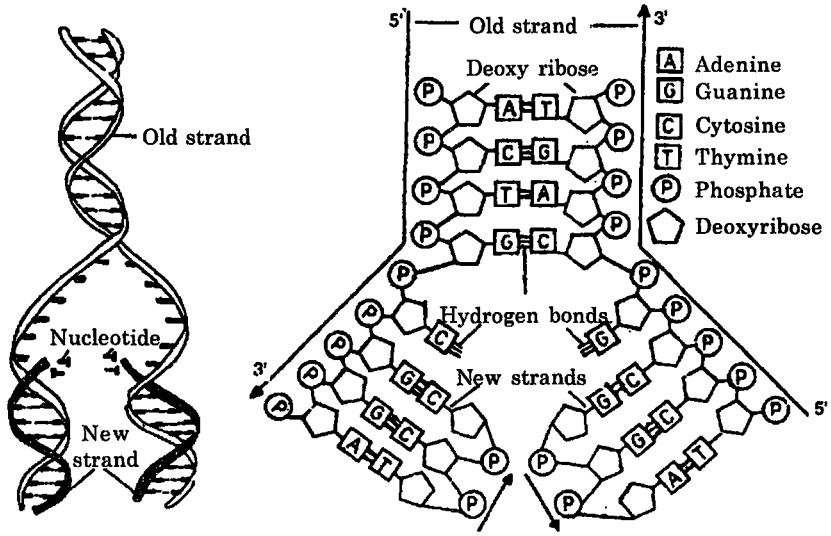


Fig. 25.28. Replication of DNA.

Outline of Replication

1. Replication takes place during the *interphase* between two mitotic cycle.

2. Replication is a *semi-conservative* process in which each of the two double helices formed from the parent double strand have one old and one new strand. Repair replication is *non-conservative.*

3. DNA replication requires a DNA *template, a primer, deoxyribonucleoside triphosphates* (dATP, dGTP. dTTP and dCTP), Mg^{2+}, DNA *unwinding protein, superhelix relaxing protein,* a modified RNA *polymerase* to synthesis the RNA primer, the products of dnaA, dnaB, dnaC-D, dnaE and dnaG genes and *polynucleotide ligase,* a joining enzyme.

4. Replication starts at a specific point called the *origin.*

5. According to one model replication starts with a 'nick' or incision made by an *incision enzyme* (*endonuclease*) (Fig. 25.29)..

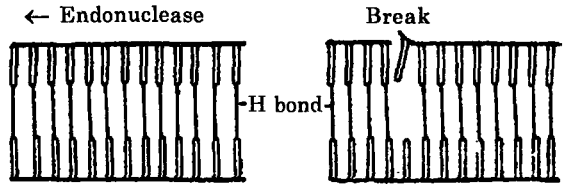


Fig. 25.29. Replication starts with an incision made by an 'incision enzyme' (endonuclease).

6. The two strands of the DNA double helix unwind with the help of a DNA *unwinding protein* (also called the DNA *binding protein*) which binds to single DNA strands.

7. The unwinding of the strands imposes strain which is relieved by the action of *superhelix relaxing protein.*

8. Initiation of DNA synthesis requires a RNA *primer. The primer is synthesized by the DNA template close to the origin of replication. The synthesis is catalysed by a special form of RNA polymerase.*

9. Deoxyribose nucleotides are now added to the 3' end of RNA primer and the main DNA strand is synthesized on the DNA template. This strand is complementary to the DNA strand is synthesized on the DNA template. This strand is complementary to the DNA strand and is synthesized by DNA *polymerase III.*

10. The enzyme DNA *polymerase I* now degrades the RNA primer and simultaneously catalyses the synthesis of a short DNA segment to replace the primer. This segment is then joined to the main NDA strand by a *NDA ligase.*

11. Replication takes place discontinuously and short pieces called *okazaki fragments* are synthesized. One strand may synthesize a continuous strand and the other Okazaki fragments, or both strands may synthesize Okazaki fragments. Both new strands are synthesized in the 5′ ⟶ 3′ direction. Thus, one strand is synthesized forwards and the other backwards.

12. The Okazaki pieces are joined by *polynucleotide ligase,* a joining enzyme, to form continuous strands.

13. Replication may be in one direction (*undirectional*) from the point of origin or in both directions (*bidirectional*).

Replication as a Semi-conservative Process

Watson and Crick were aware that any model of DNA structure should be able to explain replication. Delbruck suggested that the Watson-Crick model of DNA could theoretically replicate by three modes, *conservative, semi-conservative and dispersive* (Fig. 25.30).

(1) According to the *conservative* mode of the two double helices formed, one would be entirely of old material and the other entirely of new material. Thus the old parent double helix would be unchanged.

(2) According to the *semi-conservative* mode proposed by Watson and Crick, each strand of the two double helices formed would have one old and one new strand.

(3) According to the *dispersive* method of replication the DNA double helix would break at several points forming many pieces. Each piece would replicate, and then the pieces would reconnect at random. Thus the two double helices formed would have a patchwork of old and new pieces.

Taylor *et al.* (1957) demonstrated by autoradiography that both chromatides during prophase have half old and half new material. This indicated that DNA replication was semi conservative. The work of Meselson and Stahl (1958) has conclusively demonstrated the semi-conservative nature of replication (Fig. 25.31). *Escherichia coli* bacteria were grown for several generations in a medium containing "heavy" nitrogen (N_{15}), an isotope of nitrogen. All the nitrogen of the bacteria, including that of DNA, became N_{15}. This DNA is heavier than ordinary DNA, from which it can be distinguished by the ultracentrifuge. The labelled N_{15} cells were now grown in ordinary "light" N_{14} media, and allowed to divide several times. After the first division the DNA was extracted and all of it was found to be a hybrid ($N_{14} + N_{15}$). This hybrid was not as heavy as N_{15} nor as light as N_{14}, but had an intermediate density. After the second division two kinds of DNA were found, normal N_{14} DNA (half) and hybrid $N_{14} + N_{15}$ DNA (half). After the third division 3/4 of the DNA was normal N_{14} and 1/4 was hybrid $N_{14} + N_{15}$.

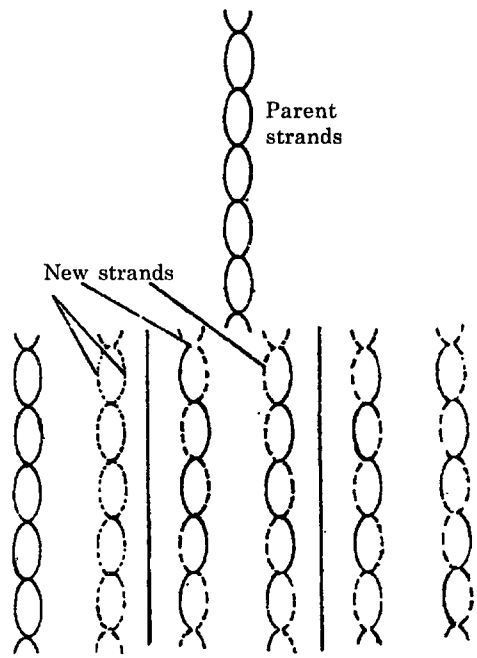


Fig. 25.30. The three theoretically possible modes of replication, conservative, semi-conservative and dispersive.

If DNA replicated *conservatively* one would expect to find two layers, one of N_{14} and the other of N_{15}, in the first generation, and similarly for subsequent generation. (Fig. 25.32). With *dispersive* replication, tubes of all generations would be expected to show a single layer (N_{14} + N_{15}), since the DNA would contain both new and old material mixed up. In *semi-conservative* replication the first generation would be expected to show a hybrid N_{14} + N_{15} layer. With each generation after the second the N_{14} layer would show a greater accumulation of material. Actual observations correspond to this expectation. This shows that *replication of DNA is of the semi-conservative manner proposed by Watson and Crick, i.e., that the double strands formed are identical to the parent strand.*

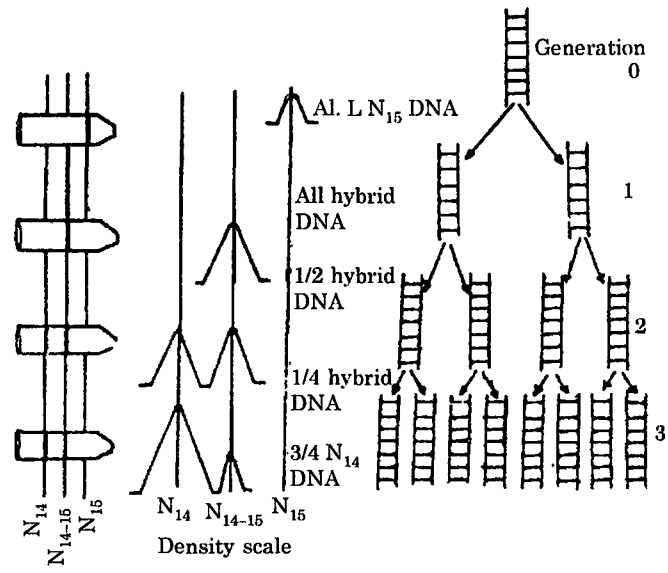


Fig. 25.31. The Meselson-Stahl experiment as evidence for the semi-conservative nature of DNA replication.

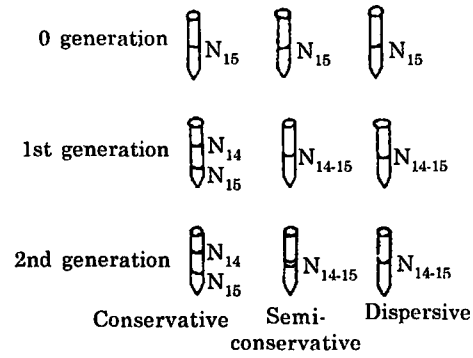


Fig. 25.32. The Meselson-Stahl experiment : expected results from conservative, semi conservative and dispersive modes of replication.

25.9 Replication of Single Stranded DNA

Many bacterial viruses are having single stranded DNA. The replication of single stranded linear molecules of DNA also takes place through base pairing on the parental strand. The parental DNA strand is generally assigned plus (+) strand of the virus. When it enters a host cell, it forms a complimentary minus (–) strand (Fig. 25.33), and hence a double helical intermediate DNA is formed. It is the replicating form of DNA because it is now acting as a template for new + strands. The + strands become ensheathed by protein molecules to produce progency viruses. Hence, the fundamental mechanism of the replication of single stranded virus DNA remains the same as that of double stranded DNA. The only difference is that in single stranded virus DNA remains the same as that of double stranded DNA. The only difference is that in single stranded viruses only one (–strand) of the two strands in the double helix of replicating form acts as template for the daughter DNA molecules.

25.10 Replication of Circular DNA

The bacterial chromosome consists of a double stranded DNA ring attached to the plasma membrane. Usually there is a single replication fork during replication. This starts at a point called the *origin and moves around the chromosome* (Fig. 25.34). During rapid growth there may be one or two additional forks formed at the origin before the first one completes its replication cycle. Several models have been proposed to explain the mechanism of replication in *circular* chromosomes. These include the *Cairns model,* the *Yoshikawa model* and the *rolling circle model.* Other models explain replication in *linear* chromosomes.

1. The Cairns model (1963) (Fig. 25.35) *:* According to this model replication begins by denaturation of the DNA double strands at a specific site called the origin. Two growing points are established and there is a bidirectional DNA synthesis. Both strands of DNA are replicated. As the growing points move apart, unwinding of the DNA double strand takes place. The unwinding creates *torque* since the parental DNA strands cannot unwind freely. The torque is transmitted to the unreplicated part of the molecule which consequently becomes *supertwisted.* Supertwisting brings about a conformational strain on the DNA molecule and prevents it from relicating further. To counteract this effect a temporary break ('nick') *is brought about on one of the strands by a swiveling protein (w).* The break permits the parental strands to rotate freely on each other, thus relieving the strain. The swivelling protein then seals the break and replication continues.

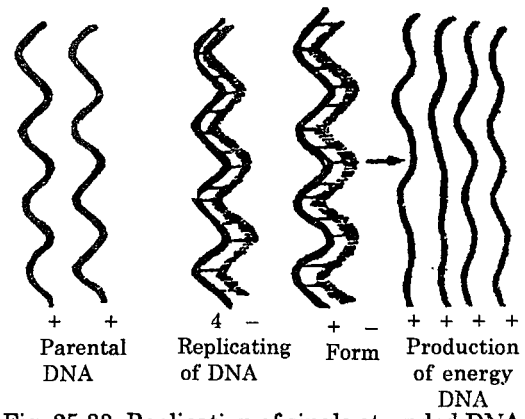


Fig. 25.33. Replication of single stranded DNA.

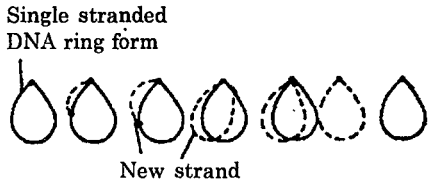


Fig. 25.34. Diagrams showing doubling of circular DNA during replication.

Cairns-type replication has been demonstrated in the bacteria *E. coli* and *Bacillus subtilis,* in several viral and plasmid chromosomes and in DNA synthesis of mitochondria and chloroplasts.

2. The Yoshikawa model (Fig. 25.36) *:* A variation of the Cairns model has been suggested by Yoshikawa. According to this model the newly formed DNA strands become covalently joined to the ends of the parental chromosomes.

3. The rolling circle model (Fig. 25.37) *:* (Gilbert and Dressler, 1968; Eisen, Pareira da Silva and Jacob, 1968) is the current model for explaining replication in single stranded DNA viruses, *e.g.,* ϕ ×174, and the transfer of *E. coli* sex factor (Plasmid).

One strand of a parental duplex ring is now cut at a specific point by an *endonuclease.* This enzyme recognizes a particular sequence at this point. As a result of the cut ('nick') a linear strand with 3′ and 5′ ends is created.

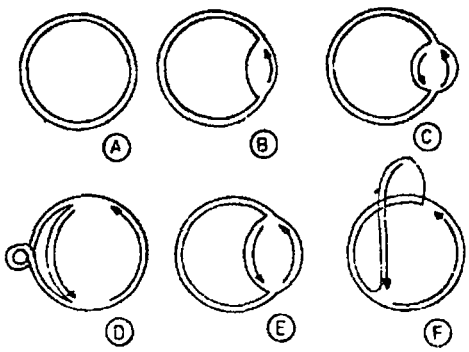


Fig. 25.35. Cairns model for circular DNA replication.

(A) double helix DNA ring.

(B) Strands denatured at origin site. DNA fragment initiated *de novo.*

(C) A second fragment initiated and a second growing point established.

(D) As a result of bidirectional replication the two growing points move forward. This causes supertwisting of unreplicated portion of DNA resulting in conformational strain on the molecule.

(E) Swiveling protein (*w*) relieves the strain by causing single-stranded nicks, thus allowing free rotation of the parental strands with reference to each other. The swivelling protein then seals the nicks.

(F) Replication proceeds and the two growing points converge on the terminus.

The 3′ end serves as a primer for the synthesis of a new DNA under the catalytic action of DNA *polymerase*. The unbroken strand is used as the template for this purpose, and a complementary strand is synthesized. Thus, the parental molecule itself is used as a primer for initiating replication.

The 5′ end of the broken strand becomes attached to the plasma membrane of the host bacterium. Such replicating phage DNA is commonly found associated with bacterial membranes.

The unbroken parental strand rolls and unwinds as synthesis proceeds, leaving a 'tail' which is attached to the membrane. New DNA is also synthesized in the tail region in discontinuous segments in the 5′ ⟶ 3′ direction. This synthesis is presumably preceded by the synthesis of a RNA *primer* under the catalytic action of RNA *polymerase*.

The tail is cut off by a specific *endonuclease* into a unit length *progency rod*. The rod may undergo *circularization* to form a new *circular molecule*. During this process the gap is closed by a *ligase*. The newly formed circular molecules can in turn become new rolling circles.

Genetic information is preserved in the single stranded template ring which remains circular and serves as an endless template. There is no swivelling problem or creation of torque in the rolling circle model. As the strands unwind the 3′ end is free to rotate on the unbroken strand. Thr growing point itself thus serves as a swivel.

Evidence for the rolling circle model has been obtained from the replication of serval viruses (M13, P2 T4, λ), replication resulting in transfer of genetic material during mating of bacteria (Fig. 25.38), and the special DNA synthesis during oogenesis in *Xenopus*.

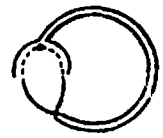


Fig. 25.36. Yoshikawa's model of replication.

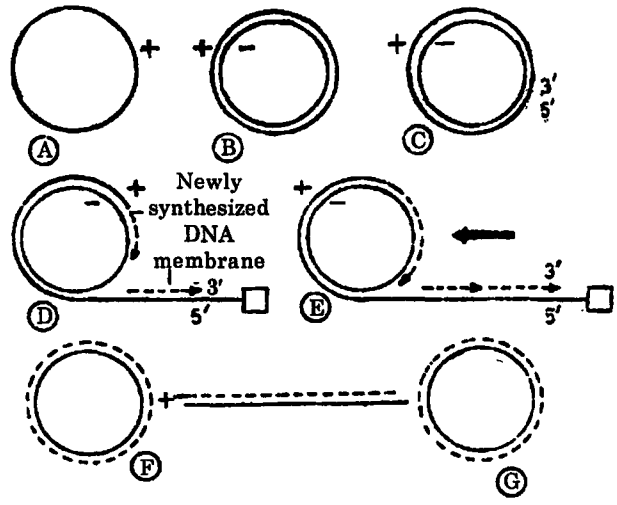


Fig. 25.37. Rolling circle model of replication of ssDNA.

(A) Single strand DNA (sDNA) ring (+) of ϕ × 174.
(B) Synthesis of negative strand (–) and formation of double-stranded (dsDNA) replicative from.
(C) Nicking of one parental strand by endonuclease.
(D) Parental strand rolls and unwinds. 5′ end attaches to the host membrane. New DNA synthesized on the 3′ end and at the 'tail'.
(E) Further unwinding and synthesis. Note that the tail region synthesizes discontinuous segments.
(F) Tail is cut by a specific endonuclease into unit length progeny rod.
(G) Circularization of rods to form new circular molecules which can becomes new rolling circles. During circularization the gap is closed by a ligase.

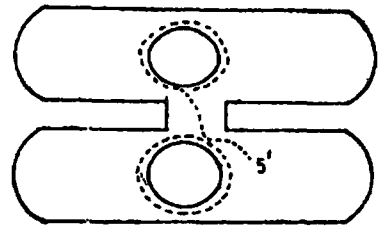


Fig. 25.38. Transfer of ssDNA during mating in bacteria through the rolling circle mechanism.

4. Replication in a duplex rod prokaryote chromosome (Fig. 25.39) *:* In the virsus T7 the chromosome is a duplex rod of about 40,000 base pairs. This size permits a coding capacity of about 25 genes. The parental strands separate at a point about 17% from one end. This site is called the *growing point.* Bidirectional DNA synthesis takes place from this point. Separation of the strands results in a replication intermediate called the *eye form.* In a more advanced stage of replication the proximal growing point reaches the end of the DNA rod, resulting in the *Y form.* Sometimes a second round of synthesis may be initiated before completion of the first round. This results in the formation of a *secondary eye* at a point 17% from the end of the arms of the Y.

5. Replication in eukaryote chromosome (Fig. 25.39) *:* In *Drosophila,* long DNA molecules are seen bearing numerous eyes growing bidirectionally. However, no secondary eye develops within the primary eye.

25.11 Synthesis of DNA in a Test Tube

The attempt to synthesize biologically active DNA in a test tube was successfully made by Arthur Kornberg and his associates (1956) who in 1954, carried out experiments by taking single stranded DNA of $\phi \times 174$ virus as a template and DNA polymerase as enzyme for cementing together the four nucleotides. The synthesis of new DNA nucleotides was detected by substituting DTTP with triphosphate of bromouracil. Bromouracil having DNA will be heavier than thymine and could be separated by density centrifugation. New DNA synthesis takes place on parental DNA template. If the enzyme polynucleotide ligase was added to the system, the newly synthesised DNA had been found to be a circle without no template activity. Addition of nucleus, however, had been found to initiate a further round of replication and the DNA synthesized was this time found to be biologically active (Fig. 25.40).

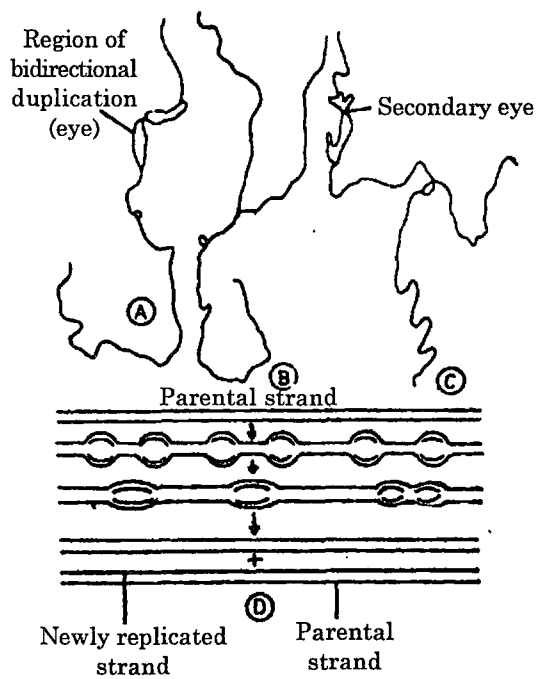


Fig. 25.39. The chromosome of the virus T7 in replication. (Tracings of electron micrographs prepared by Wolfson and Dressler. From Dressler, 1975). Although the chromosome appears like a single thread in electron micrographs it is actually a duplex rod.

(A) The early stage (eye form).
(B) A later stage (Y form).
(C) Second round of replication. Note the secondary eye on one of the arms of the Y.
(D) Replication of eukaryote chromosome.

25.12 DNA Polymerase

In 1957 Kornberg discovered an enzyme (in *E. coli*) that was responsible for the polymerization of deoxyribonucleoside triphosphates on a DNA template to form a new complementary DNA strand. This enzyme was called DNA *polymerase.* For catalysing DNA synthesis the Kornberg DNA polymerase requires the four ribonucleoside triphosphates (*d*ATP, *d*GTP, *d*TTP and *d*CTP), a DNA *template, a primer and* Mg^{2+}. In prokaryotic cells there are three DNA polymerase activities, known as *polymerase I, polymerase II and polymerase III* (also called *pol I, pol II, and pol III*). Eukaryote cells have at least three DNA polymerases, α, β, and γ.

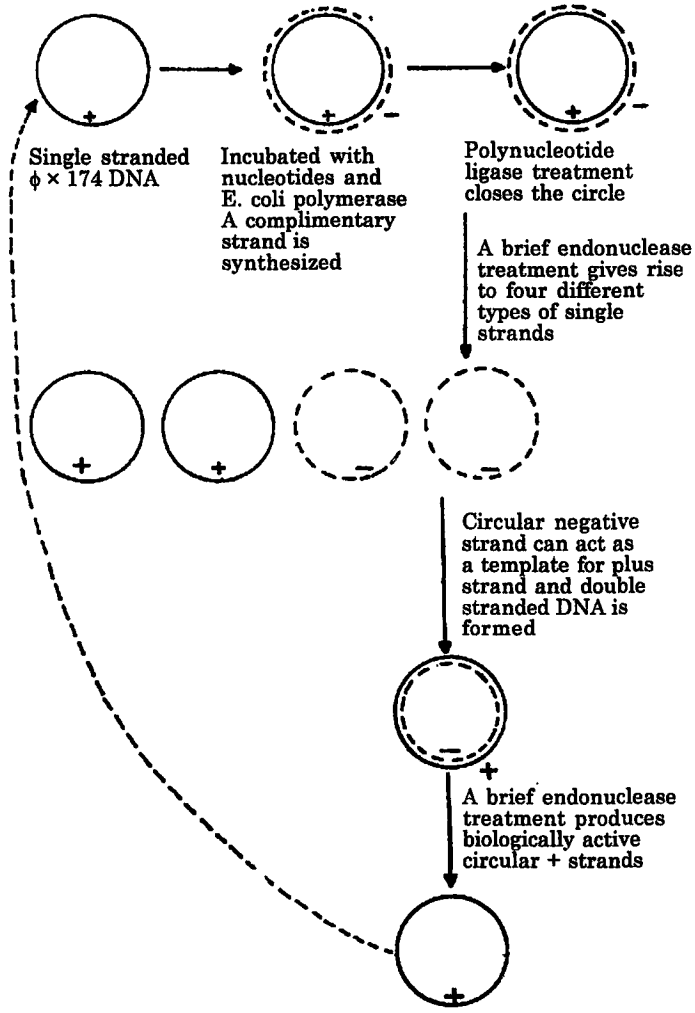


Fig. 25.40. Replication of ϕ×174 virus DNA *in vitro*.

1. Polymerase I (Pol I) (Fig. 25.41) *:* The Kornberg enzyme, now called *polymerase I,* is no longer considered to be an essential enzyme for protein synthesis. Bacterial mutants lacking this enzyme are able to synthesize DNA. It is believed to take part in the repair of DNA. Polymerase I is a single polypeptide chain with a molecular weight of 109,000. There is one atom of Zn present per chain. Thus DNA polymerases are *metalloenzymes.* About 400 molecules of polymerase I are present in *E. coli.*

Electron microscope studies show that DNA polymerase is roughly spherical in shape with a diameter of about 65A. It is attached at regular intervals to the DNA chain. DNA polymerase contains a number of sites which have functional importance.

(*i*) A *template site* which attaches to the DNA template and holds a section of DNA in place.

(*ii*) A *primer site* which contains the primer, a short (~100 nucleotides) complementary segment of RNA on which the newly synthesized DNA strand grows.

(*iii*) A *primer terminus site,* at the tip of the primer, which has a terminal 3′OH group.

(*iv*) A *triphosphate site* where an incoming nucleotide triphosphate matches a complementary nucleotide on the DNA template and is bound to the 3′OH position of the primer.

Polymerase I of *E. coli* has three activities, polymerization activity, 3′ → 5′ *exonuclease activity* and 5′ → 3′ *exonuclease activity.* (Fig. 25.42).

(i) 5′ → 3′ Polymerization : The synthesis of a new DNA chain (polymer) from its nucleotides (monomers) is called *polymerization.* The main polymerization enzyme is polymerase III, and not polymerase I as formerly thought. Pol I polymerizes nucleotides at the rate of about 1,000 molecules per minute at 37°C in *E. coli.* As previously mentioned, polymerase I is not considered to be essential for DNA replication. Mutations resulting in the absence of polymerase I do not affect DNA synthesis. It is believed that polymerase I can synthesize only short segments of DNA and takes part in *repair synthesis.* It binds to single stranded DNA and nicks in double-stranded DNA.

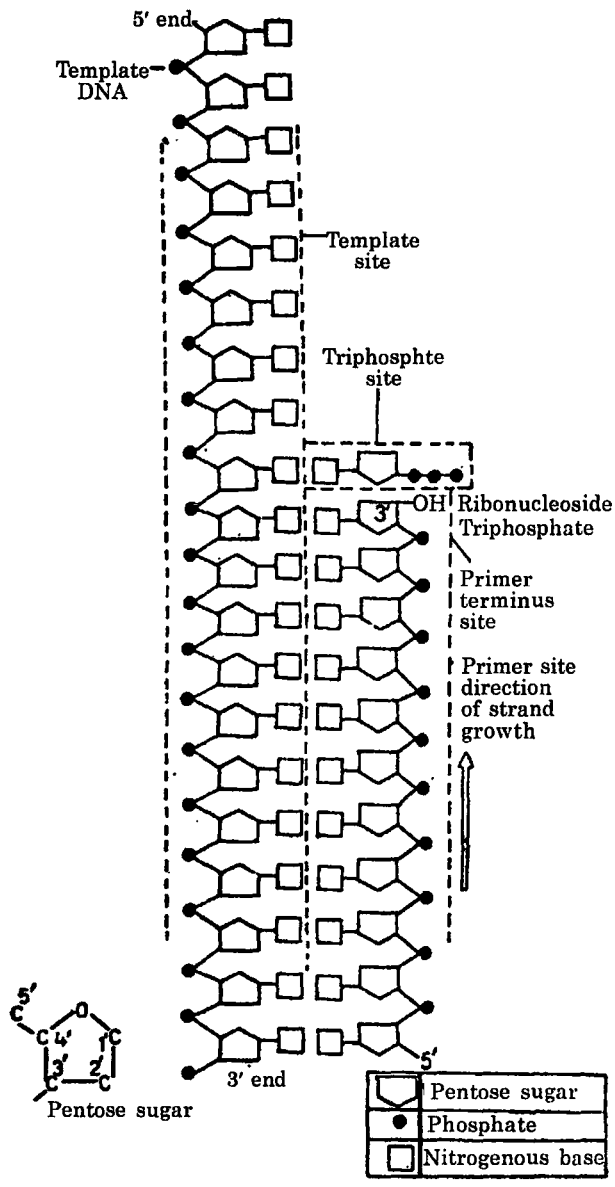


Fig. 25.41. Polymerase I.

(ii) 3′ → 5′ Exonuclease activity : Polymerase I catalyses the breakdown of one of the DNA strands into its nucleotides in the 3′ → 5′ direction. Nucleotides are removed in a sequence in a direction opposite to that of polymerization. For this reason polymerase I is called 3′ → 5′ *exonuclease.* It functions as a 'proofreader,' and 'edits' mismatched nucleotides at the primer terminus before proceeding with resynthesis of the strand. Errors made during polymerization are corrected by polymerase I. It thus acts in repair synthesis. Since base pairing is checked twice, the replication of DNA is very accurate.

(iii) 5′ → 3′ Exonuclease activity : Polymerase I can also remove nucleotides in the 5′ → 3′ direction. This 5′ → 3′ exonuclease activity has an important role in the *removal of thymine dimerse.* Exposure of DNA to ultraviolet light may result in covalent linkage of adjacent pyrimidines (*e.g.,* T = T) to form a pyrimidine dimer. Since such a dimer cannot fit into the

double helix it blocks replication unless removed. The 5′ → 3′ exonuclease activity of polymerase I excises the pyrimidine dimer region. Polymerase activity now enables repair synthesis.

Synthesis of DNA takes place in small fragments (Okazaki pieces) to each of which is attached a primer RNA segment. The 5′ → 3′ exonuclease activity is also responsible for removing the RNA segment and then filling in the gap by deoxyribonucleotides. As polymerase I moves ahead, it cuts off ribonucleotides in front and adds deoxyribonucleotides behind.

Trypsin splits the polymerase I polypeptide chain into a large fragment (MW 75,000) and a small fragment (MW 36,000). The large fragment has polymerase as well as 3′ → 5′ exonuclease activity, while the smaller fragment has 5′ → 3′ exonuclease activity (Fig. 25.43). It will thus be seen that polymerase I has *synthetic (polymerization)* activity as well as *breakdown or degradation (exonuclease)* activity.

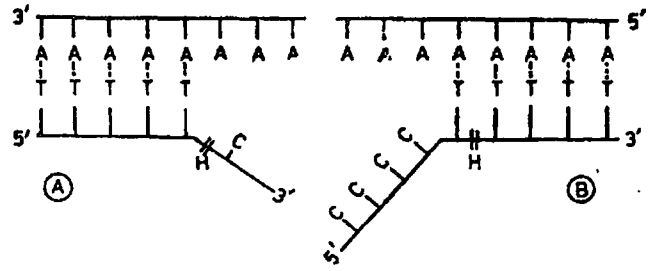


Fig. 25.42. Nucleus activities of DNA polymerase 1.
(A) 3′ ⟶ 5′ exonuclease activity.
(B) 5′ ⟶ 3′ exonuclease activity. H-Hydrolysis site.

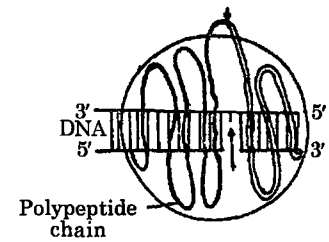


Fig. 25.43. Diagram of DNA polymerase I showing its activities. The enzyme trypsin cuts (arrow pointing downwards) the polypeptide chain into a large (black) and a small (white) fragment. The large fragment (MW 75,000) has 3′ → 5′ exonuclease activity and polymerase activity, while the small fragment (MW 36,000) has 5′ → 3′ exonuclease activity.

2. Polymerase II (Pol II) : Polymerase II is a single polypeptide chain with a molecular weight of about 90,000. There are about 40 molecules of polymerase II present in each *E. coli* cell. Polymerase II shows 5′ → 3′ *polymerization activity,* and also contains an associated *nuclease* digesting in the 3′ → 5′ direction. Unlike polymerase I, however, it *does not show 5′ → 3′ exonuclease activity.* The polymerization activity of polymerase II is much less than that of polymerase I. Only about 50 nucleotides are polymerized per minute in *E. coli.* Moreover, polymerase II can synthesize not more than 50 nucleotides of template DNA. The 3′ → 5′ exonuclease activity of polymerase II indicates that it may have an 'editing' role in *repair replication of* ultraviolet-induced DNA damage.

It has been found that polymerase II can elongate Okazaki fragments in the absence of polymerase I. It may therefore provide an alternate pathway for joining Okazaki fragments into longer DNA segments during replication.

3. Polymerase III (Pol III) : Polymerase III consists of two polypeptide chains having molecular weights of 140,000 and 40,000. Like polymerase II it has 5′ → 3′ polymerization activity and 5′ → 3′ exonuclease activity. 5′ → 3′ exonuclease activity is reported to be absent, although some works indicate that nucleotides can be removed in either 3′ → 5′ or 5′ → 3′ direction.

Recent work has shown that polymerase III is the main polymerizing enzyme. It can polymerize about 15,000 nucleotides per minute in *E. coli.* As in the case of polymerase II, polymerase III also cannot carry out polymerization efficiently if the template DNA strand is long. However, in the presence of ATP and certain protein factors, called I and II, polymerase III can synthesize DNA on long templates.

Synthesis of long templates can also take place in the presence of another polymerase, called DNA *polymerase III**, if a factor called *copolymerase III** is present. Perhaps the protein factor

II converts polymerase III into polymerase III*. The activities of polymerase III* are very similar to those of polymerase III plus the protein factors I-II.

Table 25.2

DIFFERENCES BETWEEN 3′ → 5′ AND 5′ → 3′ EXONUCLEASE ACTIVITIES

3′ → 5′ activity	*5′ → 3′ activity*
1. Removal of nucleotides (hydrolysis of DNA) takes place in the 3′ → 5′ direction.	1. Hydrolysis is in the 5′ → 3′ direction.
2. Hydrolysis begins only at the 3′-OH terminus.	2. Hydrolysis begins at the terminal phosphodiester bond or at a bond several residues away from the 5′ end.
3. The nucleotide removed must have a free 3′-OH terminus.	3. Cleavage takes place irrespective of whether the 5′ end is 5′-hydroxyl or 5′-phosphorus.
4. The products of hydrolysis are exclusively mononucleotides.	4. The products may be 5′-mononucleotides, dinucleotides or oligonucleotides.
5. The cleaved bond must be in the single-stranded region.	5. The cleaved bond must be in the double-stranded region.
6. The large fragment (MW 75,000) of polymerase I has 3′ → 5′ exonuclease activity and also polymerizing activity.	6. The small fragment (MW 36,000) of polymerase I has 5′ → 3′ activity.
7. Has a 'proofreading' and 'editing' function in the initial stages of replication. Polymerase I removes mismatched nucleotide pairs and follows this up by resynthesis.	7. Has a role in the excision of pyrimidine dimers during repair replication. (The dimers are formed when DNA is exposed to UV radiation).
8. All the three polymerases (I, II and III) show 3′ → 5′ exonuclease activity.	8. Polymerase I shows 5′ → 3′ exonuclease activity. This activity is reported to be absent in Pol II and Pol III, although some works indicate that it may be present in Pol III.

4. Characteristics of DNA Polymerase

1. In prokaryotes there are DNA polymerases, I, II and III. Eukaryote cells have three polymerases, α, β and γ.

2. DNA polymerase I has three activities : 5′ → 3′ polymerization activity, 3′ → 5′ exonuclease activity and 5′ → 3′ exonuclease activity. DNA polymerases II and III are reported to lack 5′ → 3′ nuclease activity.

3. Polymerization is the synthesis of a complementary DNA strand from deoxyribonucleoside triphosphate monomers. The strand grows in the 5′ → 3′ direction. The main polymerizing enzyme is polymerase III.

4. 3′ → 5′ exonuclease activity of polymerases, I, II and III results in degradation of one of the DNA strands in the 3′ → 5′ direction. It has an 'editing' function by removing incorrectly incorporated nucleotides.

5. 5′ → 3′ exonuclease activity results in excision of pyrimidine dimers (*e.g.*, T = T) which are formed when DNA is exposed to ultraviolet light.

6. The templates for polymerase I are optimally single-stranded regions of DNA, near double-stranded regions. Polymerases II and III, on the other hand, prefer a template of double-stranded DNA with short gaps.

5. Eukaryotic DNA Polymerases

The cells of higher eukaryotes contain at least three DNA polymerases, α, β and γ. Mitochondria from mammalian cells were considered to have a different DNA polymerase activity. Recent work, however, suggests that mitochondrial DNA polymerase may be a form of γ-polymerase. The α- and γ-polymerases have a molecular weight of over 100,000 while the molecular weight of β- polymerase is 30,000-50,000.

Table 25.3

EUKARYOTE DNA POLYMERASES

DNA polymerase	*Molecular weight*
α-polymerase	12,000 - 300,000
β-polymerase	30,000 - 50,000
γ-polymerase	150,000 - 300,000

α-*Polymerase* has been referred to as the cytoplasmic DNA polymerase, but it is probable that its *in vivo* location is in the nucleus. In animal cells the major part of α- and β-polymerase activity is located in the nucleus. β-*Polymerase* (which is located solely in the nucleus) can copy a poly (A) template but not α polymerase. β-Polymerase, however, cannot utilise poly (C) as a template. In general, β-polymerase can utilise polydeoxyribose templates like poly (dA) or poly (dC) more efficiently than polyribonucleotide templates like poly (A) or poly (C).

γ-*Polymerase :* In addition to natural or synthetic DNA templates, γ-polymerase can copy a variety of polyribonucleotides like poly (A), poly (C) etc.

Mitochondrial (mt) DNA polymerase has a molecular weight similar to that of γ-polymerase. Its activity is also reported to be similar to that γ-polymerase. If confirmed this would mean that there is no uniquely different class of mitochondrial DNA polymerases. Hence there would be three rather than four classes of cellular DNA polymerases.

25.13 Regulation of Replication of DNA

DNA replication is mainly controlled by a complex system which is made up of many parts. In absence of some proteins, DNA does not replicate. If no protein synthesis takes place in the cell, DNA replication does not continue after first round. In order to explain the regulation of DNA, a model called 'replicon' has been postulated. A genetic element like bacterial chromosome, an episome or a phage is regarded as a replicating unit or a replicon. The replicon always replicates entirely. Replication of one part of a genetic system but not of the other reveals that the system has been composed of 2 or more replicons.

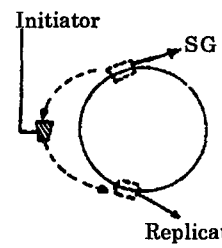


Fig. 25.44. A circular replicon.

Initiation of DNA replication takes place if the initiator molecule starts functioning. The initiator molecule gets synthesized by one of the genes of replicon and it is considered to be an integral component of the DNA polymerase. Its structure is more or less like a wedge and is contributing to the correct 3 D structure of the native enzyme. The synthesis of initiator makes the cell to replicate its DNA and to divide. It is possible to recognise initiation by only a specific sequence on the replicon called *operator of replicator or replicator* (Fig. 25.44). After the initiator gets attached to replicator, the replication of DNA starts and continues until the complete replicon is copied. The next round of replication will not take place until the cycle is complete and then the new initiator is made. The replication of DNA takes place in a fixed direction, generally clockwise.

The initiator and replicator are very specific. An initiator produced by a phage genome will get attached to replicator of phage only.

25.14 Transcription of DNA, Synthesis of RNA on DNA Template

The mechanism of the synthesis of RNA on DNA template has been found to be much similar to DNA replication. All the three types of RNAs have been found to be transcribed on DNA template. The DNA strands during transcription are separated or get melted at a fixed point and function as template on which ribonucleotides get joined together by the enzyme called RNA polymerase (Fig. 25.45). The sequence of bases in RNA is thus found out by the template DNA.

$$\begin{matrix} n\,ATP \\ n\,GTP \\ n\,CTP \\ n\,UTP \end{matrix} \xrightarrow[\text{DNA template, } Mg^{2+},\ Mn^{2+}]{\text{RNA polymerase}} (Ap)_n\,(Gp)_n\,(Cp)_n\,(Up)_n\,(Up)_n + 4n\,PPi$$

When DNA polymerase starts moving along the DNA template, the growing RNA chain gets detached and again there occurs joining of complimentary DNA chains together by hydrogen bonds (Fig. 25.45). The enzymatic transcription always takes place in a fixed direction, either 3′ to 5′ or 5′ to 3′ of the triphosphate; the latter is more common. The transcription is started at a fixed site on DNA. In *E. coli,* these sites are rich in cytosine and thymine nucleotides. Hence, all normal RNA chains get started with either adenine or guanine. During transcription, the enzyme RNA polymerase gets complexed with DNA : RNA hybrid. This complex was isolated from *E. coli*. The rate of transcription in *E. coli* has been reported to be about 43 nucleotides per second at 37°C *in vivo*. *In vitro* it is about 7 to 12 nucleotides per second.

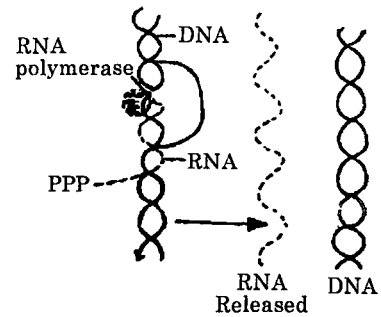


Fig. 25.45. Transcription of RNA molecule on DNA template.

The enzyme RNA polymerase was isolated and characterised by Michael Chamberlain and Paul Beg (1962). It has been found to sediment at 15s with m.w. of about 4,95,000. It is made up of several sub-units. The sigma (σ) factor constitutes an essential component of the enzyme. It is this factor which is able to recognize the DNA for initiation of transcription and forms an initiation complex. The factor gets released when the RNA chain gets synthesized. The released factor can be reemployed for further initiation of transcription. If sigma factor is lacking, the RNA polymerase (core enzyme) of bacteriophage is able to transcribe DNA but loses the ability to select the appropriate initiation sites on the template and both strands get transcribed.

25.15 Replication of RNA

If RNA viruses enter the host cells, they undergo multiplications by replicating their RNA. The mechanism has been found to be similar to the replication of single stranded DNA. In the first step the single stranded parental RNA (+ strand), undergoes replication to form a double stranded parental replicating form (+ –). In the next step, minus strands will work as templates for plus strands (Fig. 25.46). The plus strands will become part of the progeny virus particles.

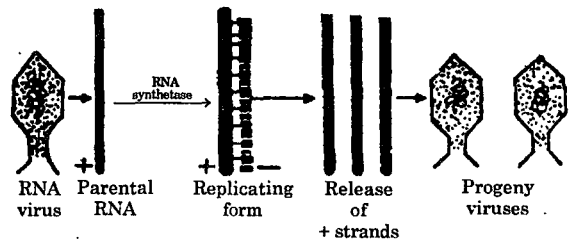


Fig. 25.46. Replication of RNA.

The enzyme which is catalysing the replication of RNA has been found to be RNA synthetase (replicase). Similar to both RNA polymerase and DNA polymerase, RNA synthetase has been found to catalyse the formation of a complimentary strand upon a single stranded template. The RNA synthetase from a RNA virus, QB, consists of 4 sub-units. The synthesis of only one of these sub-units has been coded by the viral genome while remaining three have been coded by the host genome. *In vitro* studies with replication of the virus it has clearly revealed that Mg^{2+} and two other macromolecular factors are essential for replication. These factors do not occur in QB phage but they are coming from host cell. GTP is also essential in RNA replication. The synthesis of new RNA chains from ribonucleotides takes place in 3′ to 5′ direction.

26 Biological Membranes

26.1 Introduction

All cells are bounded by a thin membrane called the *plasmalemma.* This membrane is not visible under the light microscope. The structure seen under the light microscope is the *cell membrane.* This consists of the plasmalemma along with surrounding *cell cement* (Fig. 26.1). In the broad sense the term 'cell membrane' also includes the limiting membranes of cell organelles like mitochondria and lysosomes, and other membranes like those of the nuclear envelope, the endoplasmic reticulum and the Golgi complex. These membranes, however, resemble the plasmalemma in having no cell cement.

The plasma membrane may be protected by other covering. The plasma membrane of the egg is surrounded by the *vitelline membrane* and the *jelly layer,* both of which are secreted by the ovary. In some tissues the cell cement fills the gap between adjacent cells. Fusion of the cell cement forms a supporting layer, the *basement membrane,* for a sheet of cells.

Many of cell organelles are also bounded by a membrane. The membrane around vacuoles is called tonoplast.

In some plant tissues, cells are jointed by fibrous cytoplasm which is passing through these pores in plasma membranes of the adjacent cells. The fibrous bridges are termed as *plasmodesmata.*

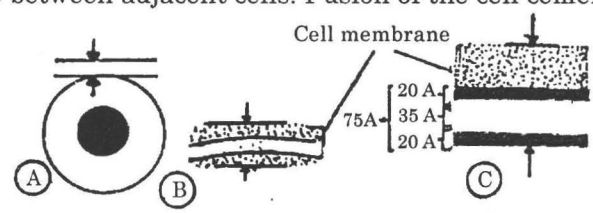


Fig. 26.1. The cell membrane : correlation between light and electron microscope views. (A) Light microscope view. (B) and (C) Electron microscope views.

In animal cells, the membranes form the outermost covering of the cell.

In somatic cells, the membranes are not lying right against one another but are separated by a space of 15 to 20 nm. The space is generally filled up with some amorphous substance.

26.2 Chemical Composition of Cell Membranes

Membranes from different sources vary in their composition. They essentially consist of *lipoproteins* which are special non-bonded combinations of *lipids* with *proteins*. In general, membranes contain about 60% protein and 40% carbohydrate by dry weight. *Carbohydrate* is usually present to the extent of 1-10% of the total dry weight in *glycoproteins* and *glycolipids*. In membranes of some cell organelles the nucleic acids DNA and RNA have also been reported, but are most likely contaminations.

1. Proteins : Membranes contain three different classes of proteins, *structural proteins, enzymes* and *carrier proteins. Structural proteins,* form the 'backbone' of the cell membrane. They have little catalytic activity and are extremely lipophilic. Their amino acid composition is nearly the same. The plasma membrane consists largely of structural proteins. The average molecular weight of structural proteins is 3×10^4. *Enzymes* form the major component of many membranes and are catalytic proteins. The endoplasmic reticulum, mitochondria, and plasma membrane contain many enzymes. The structure of the enzymes varies from membrane to membrane. *Carrier proteins* or permeases transport substances across the membrane against the concentration gradient. The molecular weight of structural and carrier proteins are rather similar.

The amount of protein varies in different cell membranes. Myelin contains only 20% protein, plasma membranes about 50% and the inner mitochondrial membrane 75%.

The plasma membrane proteins fall in two main categories, *intrinsic or integral* proteins and *extrinsic or peripheral* proteins. The former are firmly associated with the membrane, while the latter have a weaker association and are bound by electrostatic interaction. Human erythrocyte glycoproteins consist of three chemical distinct regions : (1) an N-*terminal part* external to the membrane and containing all the carbohydrate, (2) a middle *hydrophobic region located within the membrane and* (3) *a hydrophilic* C-*terminal protein,* rich in proline but lacking carbohydrate, on the internal side of the membrane. The middle intramembranous part appears to be intimately associated with the membrane phospholipids.

The glycoprotein of human erythrocyte membrane has a polypeptide backbone to which are attached two types of oligosaccharide chains, *straight chain* and a longer *branched one* at about 10 sites (Fig. 26.2).

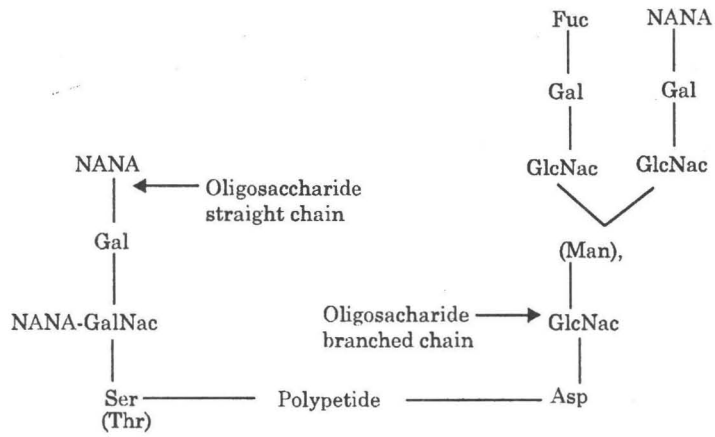


Fig. 26.2. Structure of human RBC glycoprotein (MW 50,000, 60% carbohydrate, 40% protein).

NANA.	N-acetyl neuraminic acid	Fuc.	Fucose.
Gal.	Galactose	GlcNac.	N-acetyl glucose
GalNac.	N-acetyl galactose.	Man.	Mannose.
Ser.	Serine.	Asp.	Aspartic acid.

2. Lipids : The lipids of the cell membrane are *polar lipids* which contain *hydrophilic heads* and *hydrophobic tails.* The hydrophobic and hydrophilic region may be bridged by a glycerol moiety (Fig. 26.3), *a sphinganine* derivative or homologue, or a sterol.

Sphinganine derivative also have the *hydrophobic head* attached to the primary hydroxyl group. One of the two long aliphatic chains is built into the structure. The other is attached to the *amino group by amide linkage.*

. The three main lipid constituents of cell membranes are *phospholipids, glycolipids and* sterols (Fig. 26.3).

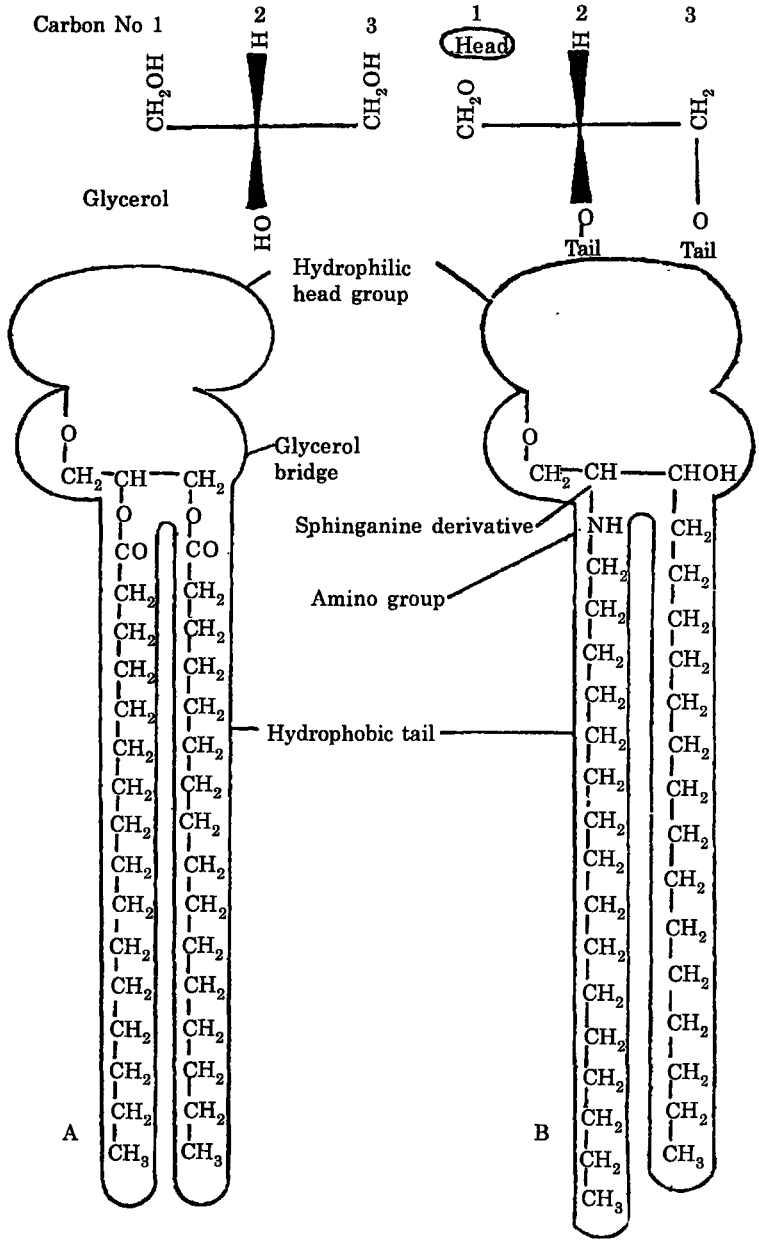


Fig. 26.3. Above Glycerol and polar lipid based on glycerol.
Below A. Polar lipid based on sphinganine (bold type).
B. Polar lipid based on sphinganine (bold type).

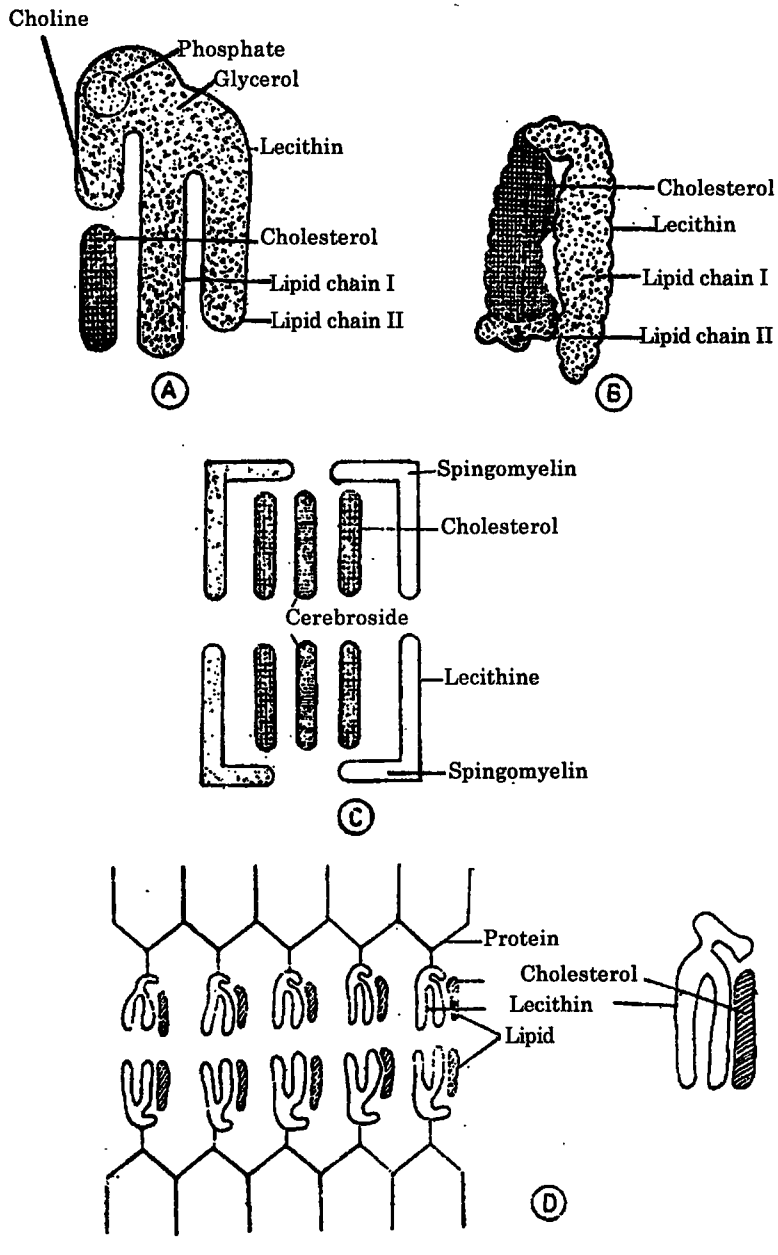


Fig. 26.4. The phospholipid-cholesterol complex of cell membranes. The arrangement of lecithin and cholesterol according to (A) Finean (1953) and (B) Vandenheuvel (1963). (C) The composition of nerve membranes. (D) A scheme for the arrangement of lipids in a membrane.

Distribution of membrane lipids

(i) Neutral fats are largely absent from cell membranes. Neutral fats such as *triglycerides* are found mainly in the cytoplasm and represent the major form of storage lipids.

(ii) Glycerophospholipids are the principal constituents of bacterial membranes. Sphinogolipids and cholesterol are absent. In animal cell membranes *glycerophospholipids and sphingophospholipids* comprise the polar lipids, while the principal *non-polar* membranes lipids are neutral sterols.

(iii) Use of chemical markers has shown that phospholipids are asymmetrically arranged in human erythrocyte membranes. The outer membrane leaflet contains the zwitterionic phospholipids sphingomyelin and phosphatidyl choline, while the inner membrane leaflet contains phosphatidyl ethanolamine, phosphatidyl serine and, when present, *phosphatidyl inositol.* The head groups of phospholipids in the inner membrane leaflet are largely *anionic.*

(iv) In rat liver membranes phospholipids comprise about 60% of the lipids in plasma membranes and Golgi membranes, about 80% in the rough endoplasmic and nuclear membranes, and about 90% in mitochondrial membranes.

(v) Phosphatidyl choline (*lecithin*) and *phosphatidyl ethanolamine* (*cephalin*) are the most abundant glycerophospholipids in higher plants and animals. Cephalin is the main phospholipid of bacteria. Lecithin is rarely present.

(vi) Phosphatidyl glycerol is present in small amounts in animal mitochondrial membranes. In plant membranes it constitutes 20–30% of phospholipids, and in chloroplasts 40–60%. The amount of phosphatidyl glycerol varies in bacterial membranes, being as high as 70% of the membrane phospholipid in Gram positive bacteria. Phosphatidyl glycerol is a precursor of *diphosphatidyl glycerol (cardiolipin)* which is found in mitochondria, chloroplasts and bacteria.

(vii) Choline plasmalogen is abundant in heart muscle. *Ethanolamine plasmalogen* is present in relatively high concentration in myelin and in lesser amount in heart muscle. In bacteria plasmalogens are confined to anaerobes.

(viii) The membranes of eukaryote cell organelles contain sterols in varying amounts. The plasma membrane is comparatively very much richer in *cholesterol.*

(ix) Cholesterol : Phospholipid ratio : In human erythrocyte membranes the outer membrane leaflet is richer in *cholesterol* than the inner leaflet. In myelin outer membrane leaflet, the cholesterol : phospholipid ratio is 3 : 7.

(x) Liver plasma membranes have a low phosphatidyl choline (lecithin) content and high phosphatidyl serin, sphingomyelin and cholesterol content.

(xi) Cholesterol controls the fluidity of fatty acid chains of phospholipids. It enhances the stability of phospholipid bilayers and also reduces their permeability.

26.3 Membrane Models

In an attempt to explain the physical and biological features of cell membranes two main categories of hypotheses have been proposed, the *bilayer models and the micellar or subunit models.* In the *bilayer models* the protein and lipid constituting the membrane are believed to occur in layers. In the *micellar models* the membrane is believed to consist of a number of similar units.

A. Bilayer Model

I. Lipid Membrane

Gorter and Grendel (1925) were the first to suggest a possible structure of the cell membrane. They studied erythrocytes and found that the surface membrane was composed of a double layer of lipid molecules (Fig. 20.4). The polar groups of these molecules were situated on the outside of these layers.

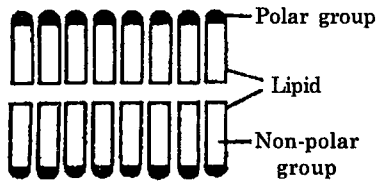


Fig. 26.4. The lipid membrane of Gorter and Grendel (1925).

II. Protein-lipid-protein (sandwich models)

1. Danielli-Davson model (Fig. 26.5 and 26.6)

By studying the surface tension of cells (Harvey and Cole, 1931; Danielli and Harvey, 1935) the

existence of protein was indicated. This led Davson and Denielli (1935) to propose a lipoprotein model of the cell membrane. According to this model the bimolecular lipid layer is similar to that proposed by Gorter and Grendel. It consists of two layers of molecules with their polar regions on the outer side. Globular proteins are thought to be associated with the polar groups of the lipid (Fig. 26.5).

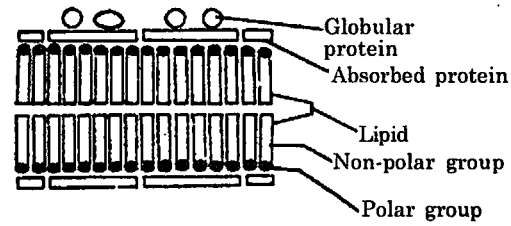


Fig. 26.5. The Danielli-Davson trilaminar sandwich model of the cell membrane.

This basic model has been modified several times. Danielli (1938) suggested that the proteins are of two types : tangentially arranged protein in contact with the lipid, and globular proteins on the outer surface. In another variation (Davson

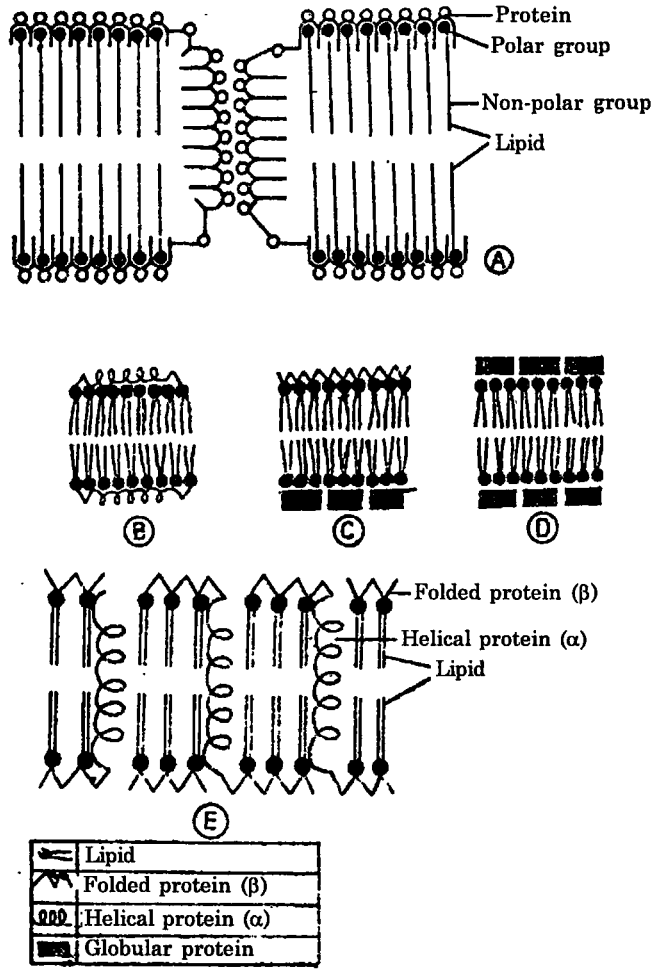


Fig. 26.6. (A) The cell membrane with protein lined pores (Danielli, 1954). (B-E) Other postulated arrangement of proteins.

and Danielli, 1943; Denielli, 1954) the proteins are considered to be in the form of a folded β chain (Fig. 26.6 A). Protein-lined *polar pores* of about 7A diameter are present in the membrane. These pores are very small and cannot be seen under the electron microscope. They probably permit the passage of small ions and water molecules. In still other variations the proteins are

thought to be in the coiled α form on both sides of the lipid layer (Fig. 26.6 B) or are thought to be asymmetrical, with a folded β-chain on the side and globular protein on the other (Fig. 26.6 C).

Models with globular protein on both surfaces (Fig. 26.6 D) or with folded protein on both surfaces and helical proteins extending into the pores (Fig. 26.6 E) are also visualized.

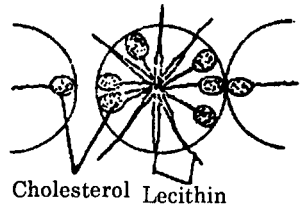


Fig. 26.7. Globular units of micelles.

Lipids in the Membrane : The lipids in the membrane consist mainly of phospholipids, with their non-polar groups near each other and their polar groups directed outwards. The lipid layer in many cases consists of a phospholipid *lecithin,* alternating with a steroid molecule *cholesterol* (Fig. 26.4 D). The *lecithin* molecule consists of two lipid chains of glycerol and a polar head containing *phosphate* and *choline.* According to Finean (1953) the head is bent around like a walking stick, and forms a bond with *cholesterol* which lies parallel to the lipid chain (Fig. 26.4A). According to another interpretation (Vandenheuvel, 1963) the second lipid chain, which is unsaturated at the ninth carbon atom curls around the end of cholesterol (Fig. 26.4 B). Thus both hydrocarbon chains lie chose to cholesterol.

Other arrangements of the lipid have been suggested. It has been found that phospholipid molecules with cholesterol form globular units or *micelles* in water. In these micelles the hydrophobic groups are packed inside, and the hydrophilic groups point outwards (Fig. 20.7).

2. Unit Membrane

In the early 1950s Robertson examined the structure of different membranes of the cell. In 1953 he put forward his *unit membrane* model. The basic unit membrane structure was considered to be general for a wide variety of plant and animal cells. Membranes of cell organelles like mitochondria and lysosomes, and other cell membranes like those of the Golgi complex, the nuclear envelope and the endoplasmic reticulum were thought to have the unit membrane structure, indicating its cellular universality.

Under the electron microscope, after osmium fixation, the cell membrane appears like two dense osmiophilic bands separated by a clear zone. The unit membrane is considered to be trilaminar, with a bimolecular lipid layer between two protein layers (Fig. 26.8 and 26.9). Each dense band is made up of protein (20A) and the polar groups of the lipids (5A), and is thus 25A thick. The clear zone is 25A thick and consists of the bimolecular lipid layer without the polar groups. Thus the unit membrane is 75A thick, with a 35A lipid layer between two protein layers, each 20A in thickness. In this respect it resembles the Danielli-Davson model. It, however differs from the Danielli-Davson model in that the protein is asymmetrical. On the outer surface is *mucoprotein,* while on the inner surface is *non-mucoid protein.* Work in the mid-1960s casts serious doubt on the universality of the unit membrane concept. The Danielli-Davson model and Robertson's unit membrane model are both based on the structure of myelin which is a non-typical membrane. It is now believed that the arrangement of lipids and proteins may vary in different membranes.

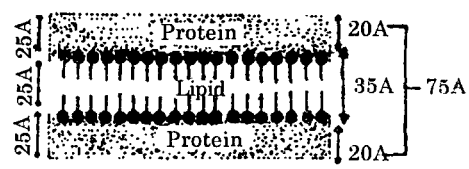


Fig. 26.8. The unit membrane.

Unit membranes vary a little in total thickness. Sometimes the membrane is asymmetric, the outer side being thicker and denser than the inner one. The plasmalemma surrounding the cell is thicker at the free surfaces of cells than where it is in contact with other cells. Also the plasmalemma is thicker than the unit membranes of the endoplasmic reticulum.

3. Greater Membrane Model (Fig. 26.10)

This model resembles the trilaminar model in that a lipid layer is sandwiched between two layers of structural proteins. Robertson, however, believed that the inner and outer surfaces of the membrane were different. The internal surface of the membrane was thought to be covered with *unconjugated protein,* and the outer surface with *glycoprotein,* which is superimposed on the structural protein. Attached to the glycoprotein are *oligosaccharide side chains* with negatively charged *sialic acid* terminals. Thus the membrane is asymmetrical and has structural polarity.

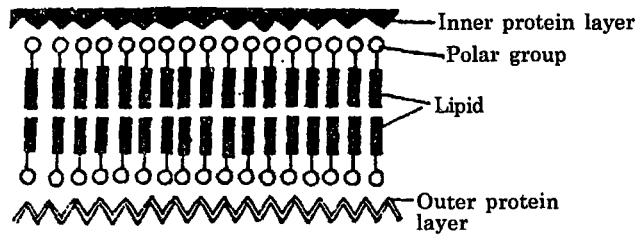


Fig. 26.9. The unit membrane (modified from Robertson, 1959).

4. Kavanu's Lipid Pillar Model

This is a modification of the Danielli-Davson model. The lipid layer exists in two forms. Under certain conditions it is in the form of *pillars,* while in other it is in the form of *flattend discs.* The space between the pillars forms pores for the passage of ions. The membrane with pillar structure is thicker than the membrane with the disc structure. The interior of each pillar is formed of the non-polar tails of the phospholipids, while the surface is formed of the polar heads. Protein layers are present on both sides of the membrane. This model cannot explain active transport or the differential permeability of NA and K ions.

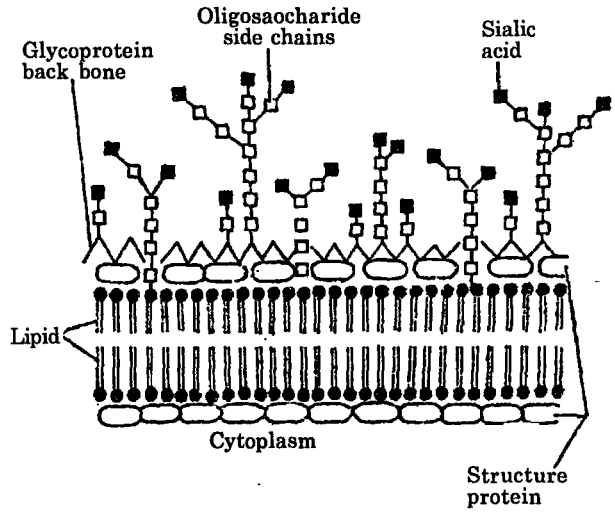


Fig. 26.10. The greater membrane model.

III. Models in Which the Proteins are Considered to Penetrate the Lipid Layer

1. Benson's model (Fig. 26.11). Benson (1966) proposed a model on the basis of a study of chloroplast membranes. The membrane lipids and protein have a hydrophobic association. The lipid tails are bound by hydrophobic regions with complementary hydrophobic regions within the interior of proteins. The charged polar heads of the phospholipids lie on the surface of the membrane and are capable of binding ions. Flexion of contractile proteins facilitates ions transport.

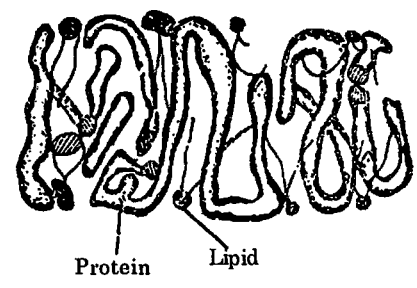


Fig. 26.11. Benson's model (1966) Protein within lipid bilayer.

2. Lenard and Singer's model (1966): According to this model one-third to one-fourth of the proteins are in *helical conformation* while the rest most likely form *random coils.* In the earlier membrane models proteins were supposed to be in extended β configuration.

3. Mosaic membrane concept : Baum (1967) considered the backbone of the membrane to be consisting of cuboidal units, 80A in diameter. Each unit of protein is toast shaped, and is covered on both sides and along the edges by phospholipids. The heads of the phospholipids stick out while their hydrophobic tails form complexes with the hydrophobic surface of the protein. In mitochondrial membranes the stalk and head are attached to the backbone.

4. Fluid-mosaic model of Singer and Nicolson (1972) (Fig. 26.12) *:* The essential feature of the fluid-mosaic model is that biological membranes are considered to be quasifluid structures in which the lipids and integral proteins are arranged in a mosaic manner.

The fluid-mosaic model of Singer and Nicolson (1972) is now widely accepted as best explaining the properties of the cell membrane (Fig. 26.12). This model assumes that there is a continuous bilayer of *phospholipid* molecules in which are embedded *globular proteins.* The proteins have been compared to icebergs floating in a sea of the phospholipid bilayer. Thus biological membranes are considered to be *quasifluid* structures in which lipids and integral proteins are arranged in a *mosaic* manner. While the Daneilli-Davson model assumes *hydrophilic bonding* between lipids and proteins, the Singer-Nicolson model considers the lipid-protein association to be *hydrophobic.* The fluidity of the membrane is the result of this hydrophobic interaction. It should be noted that the phospholipids and many intrinsic proteins are *amphipatic* molecules, *i.e.,* both hydrophilic and hydrophobic groups occur within the same molecule.

The globular proteins of the membrane are considered to be of two different types, *extrinsic (peripheral)* protein and *intrinsic (integral)* proteins. The peripheral proteins are soluble and readily dissociate from the membrane. They are entirely outside the lipid bilayer. The integral proteins are relatively insoluble and dissociate with difficulty. Some may partially penetrate either surface of the lipid bilayer, while others penetrate right through. The latter are in contact with the aqueous solvent on both sides of the membrane. The integral proteins are amphipatic. Their hydrophilic polar heads protrude from the surface of the membrane, while the non-polar regions are embedded in the interior of the membrane. The integral proteins are capable of lateral diffusion in the lipid bilayer.

When phospholipids are dispersed in water, they form a lipid bilayer. The polar heads of the lipid molecules project into the aqueous phase. The hydrophobic chains aggregate together. Studies with nuclear magnetic resonance (n.m.r.) and electron spin resonance (e.s.r.) techniques indicate that the lipid bilayer has many dynamic motional properties (Fig. 26.13).

(i) Firstly, it is possible that there is rapid internal motion involving flexing within each lipid molecule.

(ii) Secondly, a rapid lateral diffusion of the lipids is possible.

(iii) Thirdly, a slow 'flip-flop' motion, *i.e.,* a transfer of lipid molecules from one side of the bilayer to the other, is also possible.

(iv) Lastly the lipid molecules might rotate about their axes.

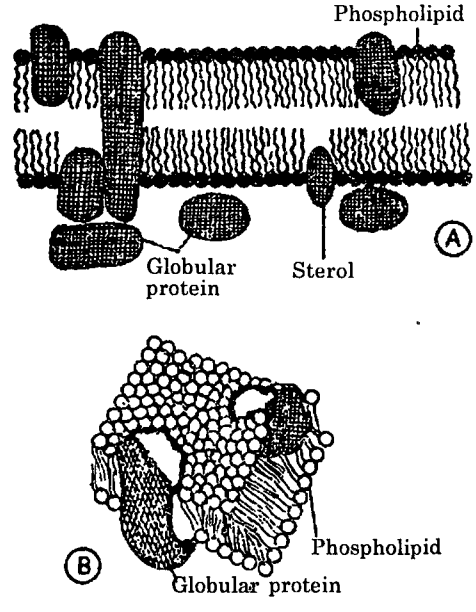


Fig. 26.12. (A) Schematic representation of a section through a eukaryote fluid-mosaic membrane. (B) The fluid-mosaic model of Singer and Nicolson (1972) : bacterial membrane.

(B) Micellar Models

Corpuscular Nature of Membrane and the Repeating Unit Concept

Fernandez-Moran (1962) and Sjostrand (1963) considered the membrane to consist of *globules units* or elementary particles. The globules, 40A-70A in diameter, form repeating units which are closely packed together. All membranes, however, do not have this structure. It is possible that both the unit membrane and the globular form are present in the cell. Under certain environmental or metabolic condition one form may give rise to the other. Some workers consider the corpuscular nature to be an artifact. On the other hand Sjostrand and Barajas (1968) hold that the typical triple nature may be the result of denaturation.

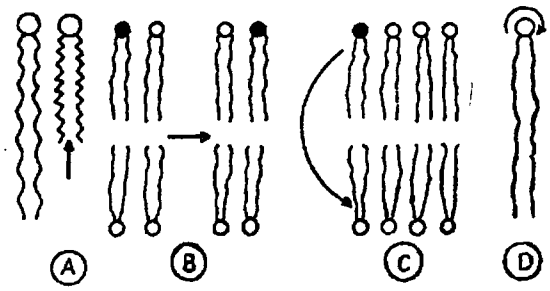


Fig. 26.13. Motional properties of the lipid bilayer. (A) Flexion, (B) Rapid lateral diffusion, (C) Flip-flop, (D) Rotation.

According to Green (1970) the cell membranes consist of fused *repeating units* (Fig. 26.14). Two types of membranes are distinguished, *monopartite and multipartite.* In *monopartite* membranes the repeating units do not have any projections. Examples of such membranes are the outer membrane of the mitochondrion and the plasma membrane of erythrocytes. In *multipartite* membranes the repeating units have projections. Each repeating unit is divisible into a base piece, a *stalk and a headpiece.* The monopartite repeating units correspond to the base pieces of the multipartite units.

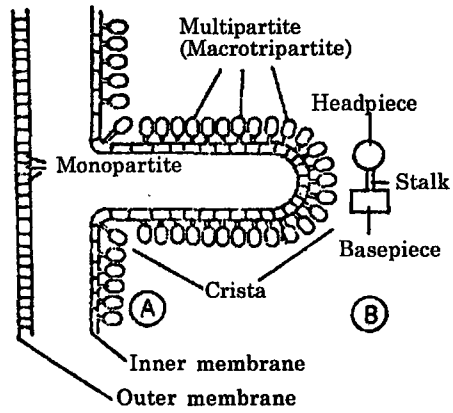


Fig. 26.14. Green's repeating unit model
(A) Crista of mitochondrion.
(B) Multipartite repeating unit.

Multipartite repeating units include *macrotripartite and microtripartite* units. The macrotripartite repeating units are larger than the microtripartite repeating units. They are found in membranes in which electron transfer is coupled to synthesis of ATP, *e.g.,* the inner membrane of the mitochondrion. Membranes having microtripartite repeating units have the capacity to carry out ATP energized active transport. The membrane of the sarcoplasmic reticulum, which transports Ca^{2+}, the plasma membrane of R.B.C. which transports K^+ and Na^+, and the microvilli which transport sugars and amino acids, all have microtripartite repeating units. In macrotripartite membranes ATPase function is localized in the headpieces, while in microtripartite membranes, it is localized in the basepieces. In microtripartite repeating units the headpiece and stalk are more firmly attached to the basepiece than in the macrotripartite repeating unit.

Bilayer and the Micellar Hypothesis : A Summation

Electron microscope studies have provided support for both the bilayer structure and the micellar (subunit) structure. Electron micrographs have clearly revealed the three layers of the unit membrane. On the other hand studies of the chloroplast and inner mitochondrial membranes show the presence of subunits in the form of repeating globules. It cannot be said definitely which

of the two cases represent the natural condition. Distortions resulting from preparation techniques could apply to either case. A possible explanation is that different membranes may show different types of structure, or that there may be transformation between the bilayer and the micellar states (Fig. 26.15). The membranes of the mitochondria and chloroplasts appear to be constructed of a bilayer backbone on which is imposed a subunit structure.

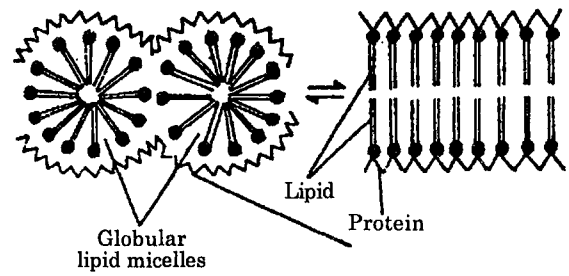


Fig. 26.15. Transformation between micellar and bilayer states of the cell membrane.

26.4 Functions of the Cell Membrane

In this section of the term 'cell membrane' is being used in the broad sense to include the membranes of various cell organelles, in addition to the plasma membrane which bounds the cell surface. Many metabolic processes occur within components of the intercellular membrane system.

(1) Transport : Cell membranes are selectively permeable rather than semi-permeable. The cell is surrounded by fluids which may be isotonic, hypertonic or hypotonic to the fluids of the cell.

The plasma membrane acts as a barrier which, however, permits the movement of certain substances into and out of the cell. Thus the membrane regulates the passage of certain nutrient molecules into the cell and the removal of waste products from the cell.

Transport of molecules across the membrane may be *active* or *passive.* Both active and passive transport involve *selectivity.* Some molecules cross the membrane more easily than others. A *carrier molecule* may also be involved in both systems.

The passage of molecules through the membrane from a high concentration to a low concentration region is called *passive transport.* Transfer of molecules takes place *along* the concentration gradient, and *no energy is required.*

In *active transport* molecules usually move from regions of low concentration, to regions of high concentration, *i.e., against* the concentration gradient. Active transport *requires energy,* and is dependent upon ATP supply in many cases. If the giant axon of the 'squid' is treated with cyanide, ATP synthesis stops and transport of Na^+ ions across the membrane is affected. Intracellular injections of ATP restores extrusion of Na^+ ions.

Transport of metabolites across biomembranes takes place in at least four ways :

Passive Transport —	*(i) Simple diffusion*
	(ii) Facilitated diffusion
Active Transport —	*(iii) Simple active transport*
	(iv) Group translocation

(i) Simple diffusion : Transport of metabolities across the membrane along the concentration gradient and without the use of a carrier molecule is called simple diffusion. Simple diffusion does not involve any stereo-specificity (*i.e.,* both L and D isomers move across at equal rates), and is a slow process. Consequently it is not believed to be an important mechanism for transport across cell membranes.

In the *Danielli-Davson model* it was assumed that passage of substances took place through small (7A) rigid protein-lined *pores in* membrane (Fig. 26.16). According to the *Singer-Nicolson*

fluid mosaic model the pores may not be stable, but may be constantly appearing and disappearing (*statistical pore concept*). They are believed to be formed by the appearance of gaps in the highly fluid lipid bilayer because of random movement of membrane through the lipids. Small polar molecules could cross the membrane through the gaps (pores) which arise in a random manner and are transitory.

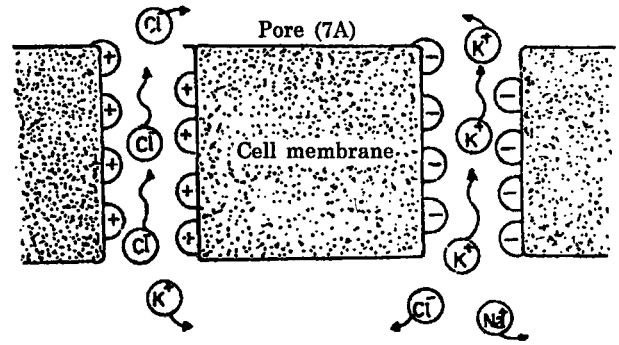


Fig. 26.16. Passage of substances through pores in a membrane.

(ii) Facilitated diffusion resembles simple diffusion in that it does not require energy and takes place along the concentration gradient (Fig. 26.17). It, however, differs in certain respects. Firstly, the process is *stereospecific, i.e.,* only one of the two possible isomers, L and D, is transported. Secondly, it shows *saturation kinetics.* Increase in the concentration of the substance to be transported results in an increase in the rate of transfer up to an asymptotic value. Thirdly, a carrier is required for transport across the membrane.

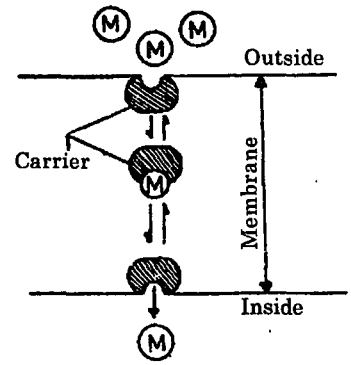


Fig. 26.17. Facilitated diffusion. M-metabolite.

The carries are proteins with a relatively low molecular weight (9,000 to 40,000). Experimental evidence indicates that the proteins are highly selective. Carrier proteins specific for individual sugars and amino acids, phosphate, Ca^{2+}, Na^+ and K^+ have been isolated. The carrier protein molecules are presumed to move to and fro across the membrane by *thermal diffusion.* The metabolite binds to the carrier protein at the outer surface of the membrane to form a *carrier-metabolite complex.* This diffuse along the concentration gradient, *i.e.,* from high concentration to low concentration regions. The metabolite is set free at the inner surface of the membrane because of the relatively low concentration of metabolite on the inner side of the membrane. Transportation of metabolite continues as long as there is a concentration gradient. The entry of glucose into erythrocytes is an example of facilitated transport.

(iii) Active transport appears to be of two general types, *primary active transport* and *secondary active transport.*

(a) Primary active transport is directly related with chemical energy (ATP) or electric energy (electron flow). Examples of primary active transport are Na^+, K^+ *translocating ATPase in mammals and proton translocating ATPase of bacteria.*

The existence of *sodium and potassium pumps* has been demonstrated in many eukaryote cells. Na^+ is pumped out of the cell by the sodium pump and K^+ is pumped into the cell by a coupled process. The two pumps apparently operate simultaneously, and in the absence of either Na^+ and K^+ movement of both Na^+ and K^+ stops.

It has been suggested that the essential part of the ion transport ATPase process is *cyclic phosphorylation-dephosphorylation* initiated by Na^+ and K^+ respectively. Na^+ and ATP bind to specific sites of the ATPase complex (Fig. 26.18). Bound Na^+ is required for *phosphorylation* of the larger subunit of ATPase. Phosphorylation of ATPase probably leads to a major conformational change resulting in a reorientation of the enzyme within the membrane, causing Na^+ to pass through the membrane. Similarly protein dephosphorylation may be brought about by bound

K^+, resulting in reversion to original conformation and K^+, resulting in reversion to original conformation and K^+ transport.

According to another view such large conformational changes in ATPase may not be taking place. Ion translocation is probably carried out by *minor conformational changes* in ATPase resulting from its phosphorylation and dephosphorylation (Fig. 26.19). It has been suggested that ATPase has *channel* through its centre through which ion transport takes place.

(b) Secondary active transport depends upon chemiosmotic energy (membrane potential and/or ion gradients). Examples of secondary active transport are the *glucose* transport system of the intestinal epithelium of mammals and the *lactose* permease system in *E. coli* (Fig. 26.20).

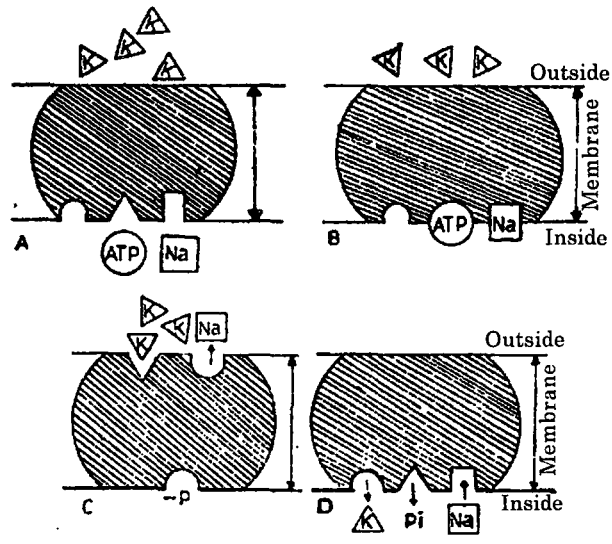


Fig. 26.18. Diagram of the Na^+ K^+ ATPase pump.
(A) Na^+ and ATP approach the inner surface of the ATPase complex (shaded area).
(B) Na^+ and the ATP bind to specific sites on ATPase.
(C) Phosphorylation of ATPase results in conformational change in ATPase and in the binding sites for Na and K.
(D) Dephosporylation of ATPase results in reversal of conformation.

The free surface of the intestinal epithelium has numerous *microvilli* which are formed by projections of the *brush border membrane.* Primary active transport results in the Na^+ being pumped out of the cell and K^+ being pumped into the cell. The electrochemical sodium ion gradient can be then utilised for *secondary active transport of glucose* into the cell against the concentration gradient. Thus there is glucose-Na^+ cotransport catalysed by a glucose carrier. Such sodium-dependent transport has been observed for various amino acids and sugars in different vertebrates and for amino acids in bacteria.

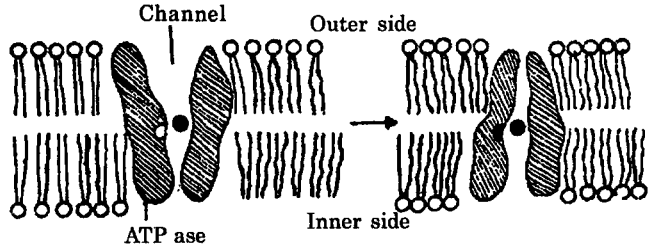


Fig. 26.19. Another model of the functioning of Na^+, K^+ ATPase.

The sodium pump maintains a higher concentration of Na^+ outside the cell than on the inner side. This results in a tendency for Na^+ to enter the cell. Thus it does in the form of a carrier-sugar or carrier-amino acid complex.

Jacques Monod and his associates studied the transport of *lactose* (milk sugar) across the cell membrane of the bacterium *E. coli.* This bacterium accumulates lactose 500-1,000 times against the concentration gradient. A 'revolving door' model of active transport has been proposed

to explain the passage of lactose through the cell membrane. The carrier, a protein, is visualized as a revolving (rotating) door in the cell membrane (Fig. 20.21). The carrier protein has a slot, which normally faces outside, into which fits the substance to be transported. The carrier protein is believed to undergo a change in shape (conformational change) when the substance enters the slot, and thereby rotates so that the slot comes on the inside. The substance is released into the cell, and the protein now returns to its original immobile form. Energy is now required to change the shape of the carrier protein, as a result of which it again rotates, so that the slot again faces outside.

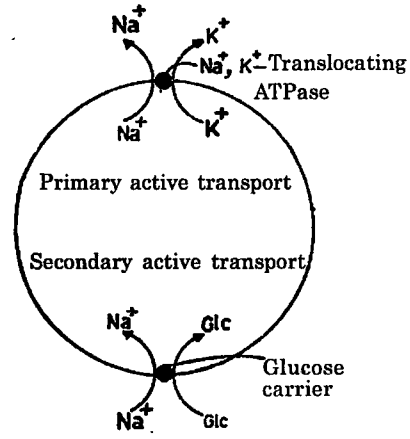


Fig. 26.20. Schematic representation of active transport in intestinal cell. Na^+, K^+ active transport at the top, and glucose Na^+ cotransport at the bottom.

Lactose transport in bacterial cells may be explained as follows (Fig. 20.22). As a result of *primary active transport protons are* pumped out through either the proton-translocating ATPase or a component of the electron transport (ETS). This sets up a proton gradient with higher concentration of protons outside the cell. This results in a tendency for protons to enter the cell. Lactose entry is now coupled to proton entry (*secondary active transport*).

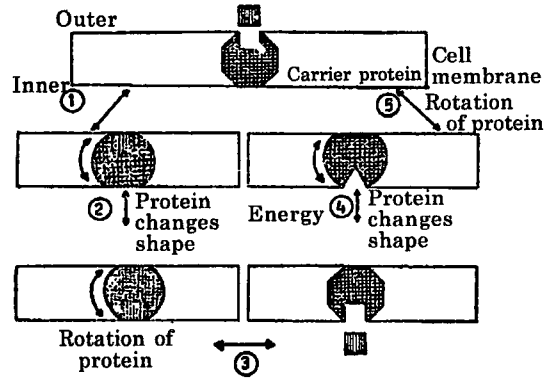


Fig. 26.21. The revolving door model of active transport.

It will be seen that there are four possible types of carrier models for transport (Fig. 26.23) :

1. Facilitated *diffusion* of the mobile carrier molecule across the membrane.

2. Ion translocation through a channel in ATPase by minor *conformational changes* in the ATPase molecule.

3. *Major conformational change* in the carrier when it attaches to the particular molecule that it normally transports.

4. *Rotation* of a carrier molecule, the diameter of which is approximately that of the membrane (*revolving door model*).

That carrier proteins are involved in certain types of transport mechanisms is well established. However the exact mechanism by which carriers transport, substances across the membrane, whether by diffusion, minor conformational changes or by rotation is not known. Large conformational changes like diffusion and rotation are probably not responsible for transport because hydrolysis of ATP and ion transport are tightly coupled processes (see Saler and Stiles, 1975). Ion translocation possibly takes place through the channel in ATPase as a result of minor conformational changes.

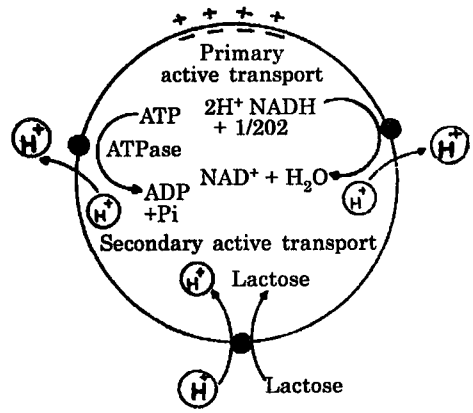


Fig. 26.22. Diagram of active transport in a bacterial cell.

(iv) Group translocation : In this process the substrate is altered by the enzyme that catalyses membrane transport. Transport of a variety of sugars like *glucose, fructose and mamnnitol* takes place across bacterial membranes through the *phosphotransferase system* (PTS). The sugar is released inside the membrane as phosphorylated derivative (*sugar phosphate*) which cannot go back through the membrane. An advantage of this system is that the sugar phosphate released can directly be utilised for metabolic activity.

The PTS always uses *phosphoenol pyruvate* (PEP) as the energy form (Fig. 26.24). Important components of the PTS are *two enzymes* (I and II) and a *heat stable protein* (HPr).

PEP is first dephosphorylated to *pyruvate* and the phosphate group released is taken up by *enzyme I,* a cytosol enzyme.

$$\text{PEP} + \text{Enzyme I} \xrightarrow{Mg^{2+}} \text{Enzyme I}\sim\text{P} + \text{Pyruvate}$$

Enzyme I then transfers the phosphate group to the heat stable protein (HPr).

$$\text{Enzyme I}\sim\text{P} + \text{HRr} \longrightarrow \text{HPr}\sim\text{P} + \text{P} + \text{Enzyme I}$$

HPr is a small (MW 10,000) *histidine-cantaining protein* also found in the cytosol.

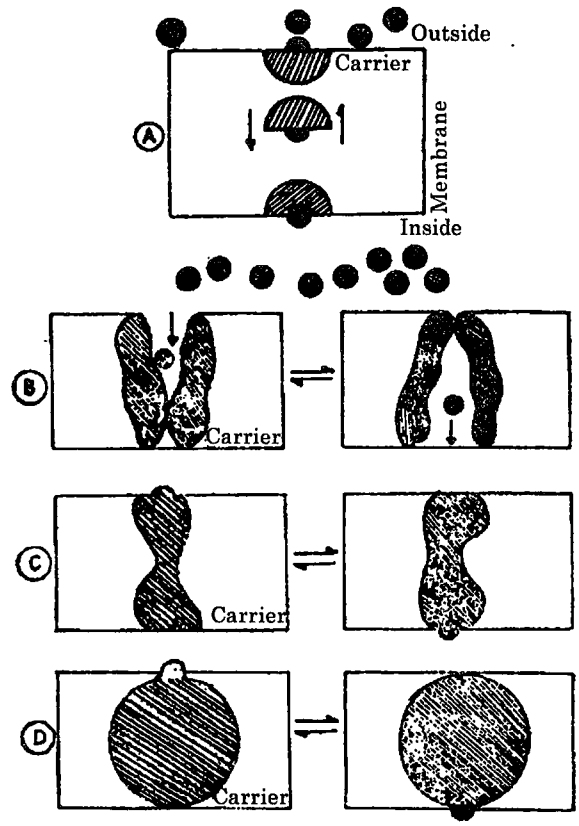


Fig. 26.23 Carrier models for membrane transport.
A. Facilitated diffusion by mobile carrier molecule.
B. Minor conformational changes in carrier molecule.
C. Major conformational changes in carrier molecule.
D. Rotation of carrier molecule.

Both enzyme I and HPR are general phosphate carrier proteins and do not show any specificity. They do not bind sugars and are hence not carrier proteins.

Enzyme II is actually an enzyme complex containing enzymes II*a* and II*b*, Mg^{2+} and *phosphatidyl glycerol.* Enzyme II*a* is *sugarspecific, i.e.,* it carries only a specific type of sugar. The cell synthesizes the enzyme specific for the type of sugar in which it is growing. Enzyme II is located in the cell membrane, probably in the outer region. It catalyses the transfer of the phosphate group from HPr to the sugar. Unlike enzyme I which is phosphorylated, enzyme II does not itself undergo phosphorylation, but only catalyses the phosphorylation of sugar.

$$\text{HPr} \sim \text{P} + \text{Sugar} \xrightarrow{\text{Enz II}} \text{Sugar-6-phosphate} + \text{HPr}$$

It is assumed that enzyme II undergoes a conformational change, thus bringing the sugar phosphate on the inner side of the membrane. The sugar phosphate is then released into the cell. HPr is then rephosphorylated by enzyme I through dephosphorylation of PEP to pyruvate.

2. Cell Recognition and Adhesion

Mammalian leucocytes recognize foreign cells like bacteria and engulf them by phagocytosis, but leave other cell types in the blood alone. Similarly macrophages of the spleen can differentiate

between healthy and worn out erythrocytes and destroy the latter by phagocytosis. The sites for cell recognition are known to lie on the surface of the plasma membrane. The amino sugar *sialic acid* is possibly involved in cell recognition and adhesion.

Reseman (1970) has proposed a theory for cellular adhesion. According to this theory the cell surface carries both *glycosyl transferases and glycosyl acceptor* molecules, which are oilgoscaccharides made up of monosacharide units (Fig. 26.25). The transferase of one cell binds with a specific receptor molecule of neighbouring cell in an enzyme-substrate reaction. Addition of a monosaccharide sugar residue to the acceptor chain is belived to bring its elongation. This results in the dissociation of the enzyme-substrate complex, and separation of the cells.

3. Antigen Specificity

The glycoproteins on the surface of the cell membrane determine the antigen specificities of the cell. The different blood group systems are all based on the antigenic properties of erythrocytic cell membranes. The ABH blood group system is based on the relationships between antigens on R.B.C. and antibodies in blood serum. The antigenic determinants on the surface of erythrocytes are mainly *glycolipids.* Those of soluble blood group substances are glycoproteins. Both M and N antigens are carried by the major *sialoglycoprotein* of the erythrocyte membrane.

A major problem in the transplantation of organs from one human being to another is tissue rejection. Transplants between genetically similar individuals are accepted, while those between genetically different individuals are rejected. This rejection is due to the immune response of the host. Recognition of foreign material depends upon specific antigens (*histocompatibility antigens*) on the cell membrane of implanted cells. The plasma membrane glycoproteins carry these antigens. If the recognition sites on the cell membranes of the cells of organs could be modified, then tissue rejection would not take place and successful organ transplantation could be carried out.

4. Hormone Receptor

It is known that hormones control the metabolism of cells. The cell membrane contains receptors which recognize specific hormones and convey the information in the latter to the interior of the cell. This stimulates a

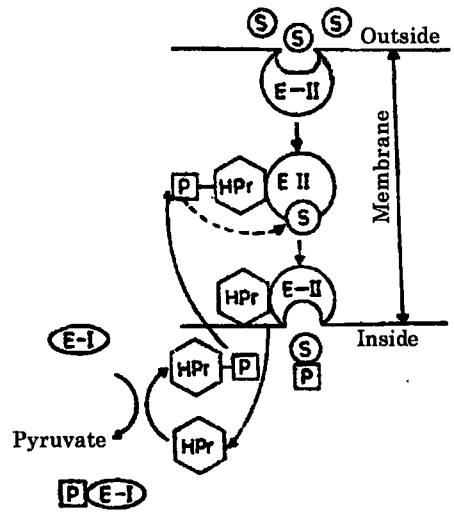


Fig. 26.24 PEP-Phosphotransferase system
PEP-Phosphoenol pyruvate.
HPr.-Heat resistant protein.
S-Sugar, P-Phosphate.
E-I-Enzyme I.
E-II-Enzyme II complex.

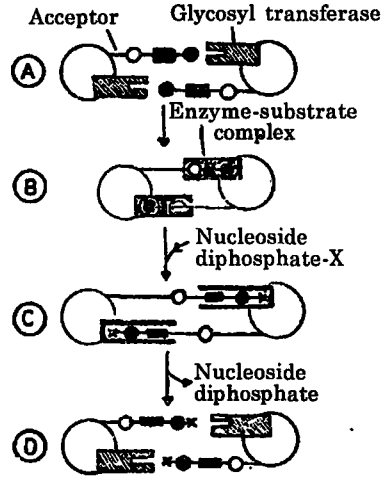


Fig. 26.25. Model for the role of glycosyl transferases in cellular adhesion.
A. Two cells, each carrying glycosyl transferases and acceptor molecules (oilgosaccharides).
B. The cells come together forming enzyme-substrate complexes.
C. One more monosaccharide residue (X) added.
D. The enzyme-substrate complex dissociates and the cells separate.

change in the metabolism of the cell. Studies have been carried out on membrane-bound hormone receptors of several hormones, including glucagon, insulin, adrenocorticotropic hormone (ACTH), angiotensin, growth hormone (GH), thyrotropin-releasing hormone (TRH), follicle-stimulating hormone (FSH), luteinizing hormone-releasing hormone (HRH), human chorionic gonadotropin (hCG) and luteinizing hormone (LH), vasopressins, oxytocin, parathyroid hormone (PTH), calcitonin, prostaglandins E (PGE), thyroid-stimulating hormone (TSH), prolactin, somatomedin, nerve growth factor (NGF), epidermal growth factor (EGF) and vasoactive intestinal polypeptide (VIP).

Location of Hormone Receptors : Hormone receptors are most commonly located in the plasma membrane, possibly on the outer surface of the membrane. The receptors for steroid hormones are, however, located in the cytoplasm. These hormones penetrate the plasma membrane and bind to the cytoplasmic receptors. The hormone-receptor complex travels to the nucleus where it influences the expression of genes.

Receptor Action : Membrane receptors have two essential functions, recognition of specific hormones and initiation of changes in the metabolism of the cell.

The first step in membrane receptor action is the recognition and binding of the hormone to the receptor (Fig. 26.26). Only a specific hormone can bind to a receptor site. The specificity is probably due to the complementary structures of the hormone and the receptor ('lock and key'). It is assumed that the binding of the hormone (H) to the receptor (R) results in the formation of a hormone-receptor complex (HR).

Hormone-receptor binding results in the activation of *adenylate cyclase,* a membrane-bound enzyme. This enzyme is believed to be located at the inner surface of the plasma membrane and contains binding sites for ATP and Mg ions. Activation of adenylate cyclase results in the generation of cyclic AMP (adenosine monophosphate) from ATP. Cyclic AMP (cAMP) *activates protein kinases* which phosphorylate a variety of *metabolic enzymes.* Depending upon the type of enzyme, phosphorylation may either increase or decrease the catalytic activity of the enzyme, and hence control cellular metabolism. Cyclic AMP thus acts as a *second messenger,* conveying information carried by the hormone (*first messenger*) from the plasma membrane to the interior of the cell. The receptor may be coupled to the enzyme by an intermediate *coupler,* possibly phospholipids.

Adenylate cyclase may be stimulated by many different hormones. For example, the adenylate cyclase of the fat cell is regulated by seven different hormones and inhibited by three. It is difficult to imagine 10 separate receptors having physical association with adenylate cyclase. In the model proposed by Cautrecasas (1974) this difficulty has been resolved (Fig. 26.27). According to this model, the receptor by itself does not have an affinity for adenylate cyclase. However, the hormone-receptor complex formed by association of the hormone with the receptor has great affinity for the enzyme. The HR complexes are supposed to diffuse laterally till they collide at random with adenylate cyclase and bind with it. This brings about a modification in the catalytic site of the enzyme, resulting in the generation of cAMP.

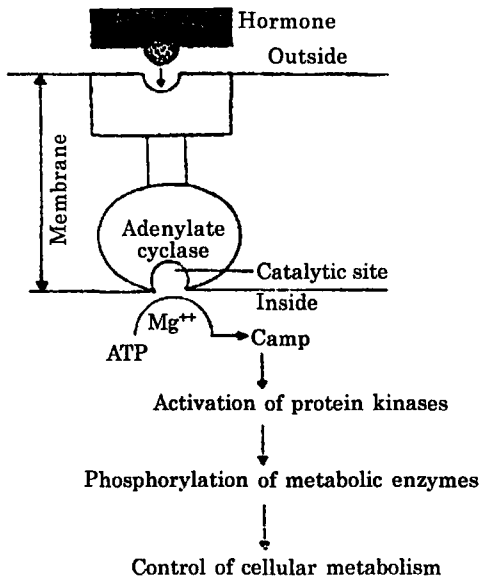


Fig. 26.26. Receptor adenylate cyclase action.

5. Secretion

Polypeptide chains are synthesized by ribosomes and released into the lumen of ER cisternae (Fig. 26.28). Secretory protein is next transported to the Golgi complex where it may undergo modification (*e.g.*, glycosylation in case of glycoproteins). Secretory vesicles released from the Golgi complex move to the periphery of the cell and fuse with the plasma membrane. This fusion apparently depends upon the interaction between the integral proteins of the plasma membrane and the secretory vesicle. Release of secretory products therefore ultimately takes place through the plasma membrane.

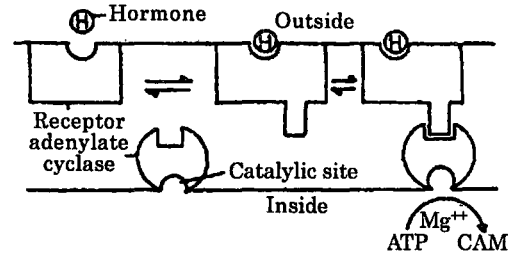


Fig. 26.27. Cautrecasas model (1974) of receptor action.

A. Hormone binds to receptor.
B. The hormone-receptor complex formed has high affinity for adenylate cyclase.
C. The hormone receptor complex may collide with adenylate cyclase by lateral diffusion and bind with it. The catalytic site of adenylate cyclase is modified, resulting in the generation of cAMP.

6. Oxidative Phosphorylation

The inner membrane of the mitochondria and the plasma membrane of bacteria contain the *electron transport chain* which plays an important part in cell respiration. This chain consists of a series of components which can transfer electrons. Electrons from substrates pass down the chain resulting in reduction and oxidation of each component. During this process there is a decrease in free energy, about half of which is utilised for the synthesis of ATP. This process is called *oxidative phosphorylation.*

7. Endocytosis and Exocytosis

An important activity of the plasma membrane of certain cell type is *endocytosis.* Endocytosis is the process by which material is transported into cells by formation of vesicles. The term includes two essentially similar processes, *phagocytosis* (cell eating) and pinocytosis (cell drinking). Phagocytosis is the bulk injection of solid food into the cell, while pinocytosis refers to the injection of fluid material. The reverse process by which membrane lined material is removed from the cell is called *exocytosis.*

Phagocytosis (Fig. 26.29) involves folding of the plasma membrane around the material that is being engulfed and the subsequent formation of an intracellular vesicle (*phagosome*). The phagosome fuses with a *primary lysosome* to form a *secondary lysosome* in which the food material is digested by enzymes. Phagocytosis is found in many protozoa, where it serves for the nutrition of the cell. In the metazoa it is a method of defence against foreign bodies like bacteria dust and various colloids, *e.g.*, in scavenging white blood cell. Residual undigested material is ejested by *exocytosis.* Injestion of small colloidal particles is sometimes called *ultraphagocytosis.* Phagocytosis is found in the granular WBC and in cells of the reticulo-endothelial system (histocytes, reticular cells, endothelial cell).

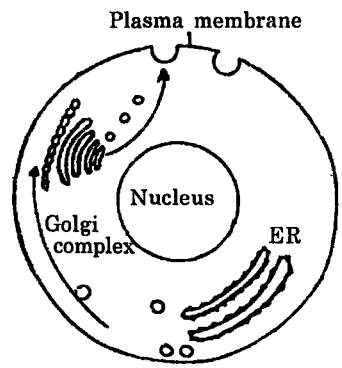


Fig. 26.28. Role of plasma membrane in secretion.

Pinocytosis (Fig. 26.30) is the intake of fluid material into the cell by the formation of *pinocytic vesicles or pinosomes.* The fluid may contain organic molecules or other nutrient material in solution. Experiments using labelled proteins have shown that the amoeba injests ('drinks') about one-third its

volume of protein solution during the feeding period. The protein apparently acts as a stimulus to pinocytosis. If an amoeba is placed in water, or in water to which carbohydrate has been added, no pinocytosis takes place. However, if placed in water to which proteins, certain amino acids or ions are added, pinocytosis begins.

Pinocytosis provides the cell with additional interior interface where active and passive transport can be carried out. It is therefore a process which supports transport of materials into the cell.

Both phagocytosis and pinocytosis are active mechanisms which require energy. This is shown by the fact that glycogen breakdown, glucose uptake and oxygen consumption all increase during phagocytosis by leucocytes.

Exocytosis is process which is essentially the reverse of endocytosis. The membrane of a cell vesicle fuses with the plasma membrane and releases its contents outside the cell. Exocytosis serves as a means of extruding undigested material from the cell. It is also the principal mechanism by which hormones and other substances are secreted from the cell.

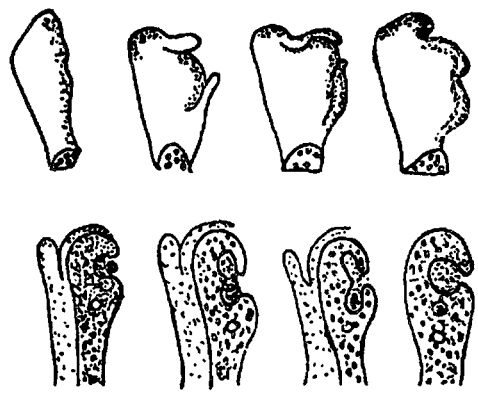


Fig. 26.29. Phagocytosis.

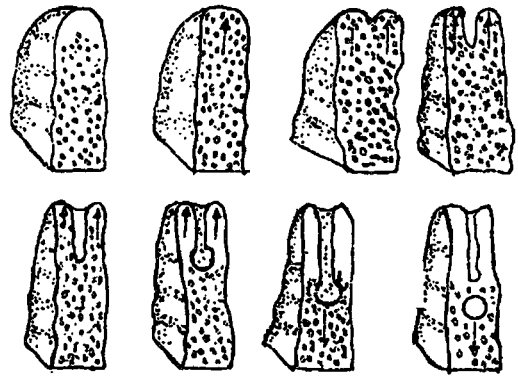


Fig. 26.30. Pinocytosis.

8. Chemoreception

Molecules associated with the cell membrane respond to a variety of stimuli. Chemoreception or the response to chemical stimuli is shown by a variety of organisms ranging from bacteria to mammals. The bacterium *E. coli* has chemical receptors, which appear to be proteins, involved in membrane transport in its membrane. Bacteria and protozoa are attracted to compounds like amino acids, sugars, and organic acids, and repelled by hydrophobic compounds, phenolic compounds and acids.

The chemical receptors in the membrane of bacteria appear to transport proteins in some cases. Thus the sugar transporting *phosphotransferase system* also appears to be associated with chemotactic response. Blocking any one component of the phosphotransferase system blocks both sugar transport and chemotaxis. This suggests that passage of sugar through the membrane is required before a substrate elicits a chemotactic response.

In mammals, binding proteins associated with the cell membranes are believed to serve as chemical receptors for taste and smell. It is believed that in man a different receptor protein recognizes each of the four distinct taste qualities : sour, sweet, bitter, and salty. A protein which binds to sweet compounds has actually been isolated. Specific receptor proteins have also been postulated for membranes of olfactory receptor cells.

9. Transmission

The transmission of nerve impulses takes place at the surface membrane of nerve cells.

26.5 Mechanism of ATP Synthesis

The main function of mitochondrial and choloroplastic membranes is the synthesis of ATP. Energy is liberated during the electron transport in the oxidation of NADH in mitochondria or in transfer of electrons from water to $NADP^+$ in illuminated chloroplast. The energy liberation takes place due to the difference in redox potentials of electron donating and electron receiving components. A part of this energy is consumed in the synthesis of high energy ATP molecules from ADP and Pi. This synthesis takes place on the inner surfaces of the membranes.

The following three hypotheses have been postulated regarding the mechanism of ATP synthesis.

1. Chemical hypothesis : This was proposed originally by E.C. Slater (1963). This model was mainly postulated to explain the substrate level phosphorylation. According to the hypothesis, there are some definite chemical compounds which take part in phosphorylation. The energy of electron transfer from one substrate to the other is absorbed by these compounds. Then these energized compounds activate inorganic phosphate which in turn combines with ADP to form ATP. The sequence of reactions may be put as follows :

$$AH_2 + B + I \rightleftharpoons A\sim I + BH_2$$

$$A\sim I + X \rightleftharpoons A + X\sim I$$

$$X\sim I + Pi \rightleftharpoons X\sim P + I$$

$$X\sim P + ADP \rightleftharpoons X + ATP$$

where AH_2 and B represent two substrates and X and I the hypothetical intermediates.

2. Conformational hypothesis : This model was originally postulated by P.D. Boyer (1967) to explain ATP synthesis. According to this hypothesis, when the energy of electron transport is taken up by a macro-molecule or a protein of the membrane, there occurs a conformational change in the macromolecule or membrane (Fig. 26.31). Then, the activated macromolecule with changed configuration relaxes thereby releasing the energy for ATP synthesis (Fig. 26.31).

Green and Ji (1972) suggested that conformational change is being induced by an electric field which is developed by the electron transport chain.

3. Chemi-osmotic hypothesis : According to Peter Mitchell (1961, 1968) electron transport could be coupled to phosphorylation of ADP by a process called chemi-osmotic process. This hypothesis has been found to be applicable to both, photophosphorylation in the chloroplast and oxidative phosphorylation in the mitochondria. According to this hypothesis, there occurs the involvement of enzyme ATPase and a well organised membrane. In the reversible reaction

$$ADP + Pi \overset{ATPase}{\rightleftharpoons} ATP + H_2O$$

the removal of water from active centre of ATPase will favour synthesis of ATP in accordance to law of mass action. This assumption forms the basis of Mitchell's chemi-osmotic hypothesis. Later on

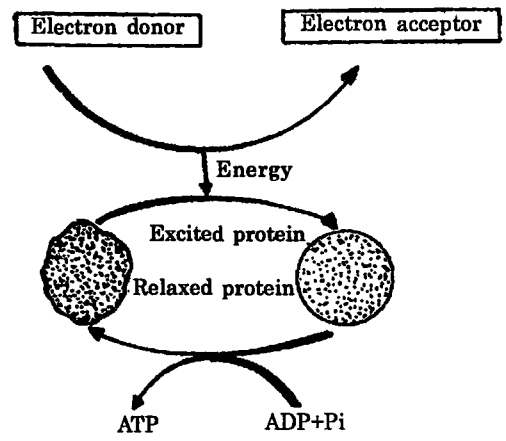


Fig. 26.31. Involvement of a structural protein in ATP synthesis (mechanical hypothesis).

the involvement of chemical intermediates X and I was also postulated. The details of mechanism are depicted in Fig. 26.32.

In ATP synthesis the electron transport in the membrane involves the two types of effects :

(*i*) Water is ionised, followed by the separation of H^+ and OH^- ions.

(*ii*) From two unknown chemical intemediates X and I, there occurs the formation of high energy X ~ I complex.

On the outer surface of the membrane, positively charged hydrogen ion combines with the complex $X - I_0^-$ and H_2O is liberated. Later on, X and I form complex which on absorbing energy liberated during electron transport is converted into a high energy X ~ I. Now X ~ I moves towards the innerside of the membrane where it undergoes combination with ADP, Pi and OH^- to form ATP and H^+ on the innerside of the membrane. The complex XI_0^- then moves again to the outer side of the membrane to combine with the hydrogen ion. In this way, this process is producing one ATP molecule in each cycle on the inner surface of the membrane. Under conditions, where ATP synthesis does not take place, the ejection of proton $(H)^+$ gets balanced by uptake of Ca^{2+} For every pair of proton there occurs the absorption of one Ca^{2+} inside.

In chemisosmotic hypothesis the involvement of two chemical intermediates (of unknown chemistry), as in case of chemical hypothesis is also required. In chemiosmotic hypothesis, however, these chemical intermediates do not have to undergo combination with an electron carrier in the respiratory chain. Also, the propounders of chemiosmotic hypothesis have been found to be much more flexible about the chemical nature of X and I than those of chemical hypothesis, who postulated that these are somewhat discrete covalent compounds.

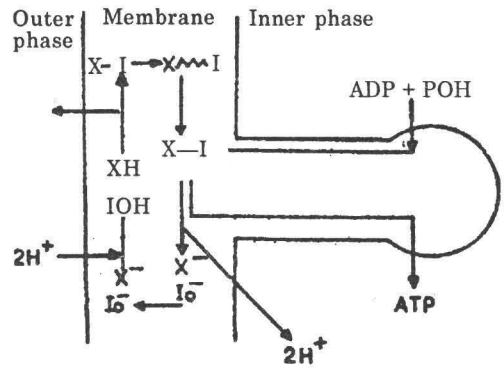


Fig. 26.32. Mitchell's modified scheme of chemiosmotic hypothesis.

Chemiosmotic hypothesis has been found to explain most of the experimental observations. The production of pH gradient across membrane, as postulated by this hypothesis, is frequently seen in phosphorylation. The pH of the medium having actively phosphorylating isolated mitochondria decreases. This decrease can be terminated by adding antimycin which is an uncoupler of phosporylation. Further, synthesis of ATP in isolated mitochondria from rat liver or beet roots has been stimulated by lowering the pH of the medium. This stimulation does not take place in the presence of dinitrophenol or oligomycin.

Although many experimental observations favour this hypothesis, it could not be accepted as an universal model of ATP synthesis. More experiments should be carried out to elaborate each step of the model, and hypothesis needs further testing.

27 Regulation of Biochemical Reactions

27.1 Introduction

A normal cell is just like a complex chemical factory which is able to make best use of available resources. The two important biochemical functions of a cell are as follows :

(i) The cell provides chemical substrates for carrying out various reactions.

(ii) The cell provides necessary energy to drive reactions.

The cell is able to carry out the above-mentioned functions very efficiently even under diverse environmental conditions. The intermediate products of cellular metabolism do not get accumulated in the cell under ordinary conditions. However, they get accumulated in the cell when an inhibitor is present or there occurs a genetic block by mutation. The energy liberated at any instant in cellular metabolism is slightly above the level needed for driving the biochemical reactions.

It is of interest to note that the demand for a particular substrate or a particular level of energy may not remain same during the entire life history of an organism. The following examples illustrate this view point.

(i) The physiological requirements of pregnant woman will be entirely different than those of an adolescent child.

(ii) The physiological requirements of a plant growing in a desert oil will be altogether different than those of a plant growing near a pond.

From the above examples, it implies that some biochemical reactions at certain stages of development or under certain environmental conditions have to be accelerated or suppressed so

as to attain a specific physiology. Further, the regulation of biochemical reactions takes place to achieve coordination among different functions.

27.2 Importance of Regulation of Biochemical Reactions

The main functions of regulation of biochemical cellular level are as follows :

(i) It becomes possible to avoid unnecessary expenditure of energy.

(ii) The cell sap may possess limited capacity to dissolve the metabolites. Therefore, the cell sap is maintained below separation level by regulating the concentration of metabolites.

(iii) Some intermediate compounds, which may be used in other metabolism, are conserved.

27.3 Determination of the Rate Limiting Step

The overall activity of a series of biochemical reactions depends on one or two steps in the series. The step or the reaction which has been found to control the activity of the series is termed as the *rate limiting step or pace-maker reaction.* An example is that in a series involving reduction of nitrate and incorporation of amino nitrogen into amino acids and proteins, the rate determining step is assumed to be the reduction of nitrate to nitrite (Beevers and Hageman, 1969). When the reduction of nitrate gets accelerated, the synthesis of various amino acids also gets accelerated. In order to ascertain the rate limiting step in a series, the following principles are used :

(a) Identification of the equilibrium of different steps : Suppose we consider a hypothetical reaction which converts substrate A to a product F involving the intermediate compounds B, C, D and E.

$$A \rightleftharpoons B \rightleftharpoons C \rightleftharpoons D \rightleftharpoons E \rightleftharpoons F$$

If these steps are reversible, then the concentration of all the intermediates at equilibrium will be more or less equal. From the measurements of equilibrium constants of different steps, an idea of the relative concentrations of different intermediates can be obtained. The step far from equilibrium normally ascertains the rate limiting step.

When apparent equilibrium constants of various steps in glycolysis are determined, it was seen that the constant for conversion of fructose 6-phosphate to fructose 1, 6-diphosphate was far from equilibrium. Various other techniques also show that this step is rate limiting in glycolysis.

(b) Determination of enzyme activity : In a series of biochemical reactions, each step is catalyzed by an enzyme. Therefore, the determination of the activities of different enzymes, may decide the rate limiting step. First of all, the crude homogenates of the cells are prepared. Then, various activities of different enzymes assayed under varying conditions of substrate concentration, pH and temperature are determined. The enzyme having the lowest rate of activity may be the pacemaker. When the activities of enzymes of glycolysis in rat liver homogenates are studied the activity of phosphofructokinase came out to be the lowest. In other tissues, such as kidney, skeletal muscle and heart also, the level of this enzyme was found to be the lowest. This revealed that the rate limiting enzyme in glycolysis is phosphofructokinase.

After isolation, the critical enzymes are purified and their optimum activities are measured. Further studies on regulation of the reaction are also carried out with these enzymes.

(c) Application of cross over principle : When cross over principle was applied to a series of reactions it was found to be probably the most definite technique for knowing the pacemaker reaction. In the hypothetical example of conversion A to F as given above, it was found that if the first step (conversion of A to B) is rate limiting, exogenous supply of A does not have any effect on the production of F. However exogenous supply of B will increase the production of F.

By supplying B the limiting step is bypassed. If a step beyond the synthesis of B is limiting, supply of B does not have any effect on the final product. This technique has been found to be very successful in determining the rate limiting step in many reactions, in which the details of pathway are known. The electron transport sequence in the mitochondria has been ascertained by this method.

27.4 Mechanism of Control

(a) Law of mass action : When the law of mass action is applied to a reversible process, it is found that the accumulation of products inhibits their own synthesis. Now the following reaction is considered :

$$A + B \rightleftharpoons C + D$$

If C and D are accumulated, then the conversion of C and D into A and B will be inhibited. However, if C and D are removed from the reaction, A and B will react to form more C and D. This type of control has been found to be able to maintain equilibrium conditions in many biochemical reactions. An example of this type of control can be seen in the formation of creatine phosphate (Lohmann's reaction).

$$\text{ATP} + \text{Creatine} \rightleftharpoons \text{Creatine phosphate} + \text{ADP}$$

The accumulation of creatine phosphate takes place till ATP is in excess. If ATP is limiting, then creatine phosphate combines with ADP to form ATP.

(b) Substrate sharing : If two or more reactions employ the same metabolite or cofactor, the availability of metabolites in those cases ascertains the rates of the reactions. An example is α-ketoglutarate which is an intermediate metabolite of Kreb's cycle as well as of glutamate synthesis. In the presence of ammonia, α-ketoglutarate is converted into glutamate. Hence, if ammonia is present in sufficient supply, Kreb's cycle gets suppressed.

An example showing the competition for the same cofactor that of reduced ferredoxin which takes part in the reduction of both CO_2 (through NADPH) and nitrate. Many biochemists have reported that when CO_2 is being assimilated either by isolated chloroplasts or by detached leaves, there occurs a decrease in the apparent rate of photosynthesis. Ferredoxin which is produced in the light is used up in the reduction of nitrite and very little reduction of CO_2 takes place.

27.5 Regulation of Enzyme Activity

From the study of many biochemical reactions, it was found that the pace of reaction has been maintained by the activities of the enzymes. Enzymes are somewhat unstable molecules. They are synthesised and degraded simultaneously. It is possible to regulate their activities either through their synthesis or by modifying the existing enzyme molecules. The following methods are used to regulate the activities of enzymes molecules :

1. Allosteric regulation : It is a fine mechanism to control a reaction through the enzyme activity. There are certain enzymes which show sigmoidal curve between the substrate concentration and the activity. These enzymes are known as *allosteric enzymes.* Several metabolites are known which are able to modify the activity of these enzymes. The effect of different concentrations of 'activator' and 'inhibitor' on these enzymes has also been found to be sigmoid. The structure of effector molecules has been found to be different from the substrate molecules.

In many cases, the end products of the reaction are allosteric inhibitors, thereby inhibiting the first enzyme in the series. Hence, this kind of inhibition is termed as *feed back inhibition, end product inhibition or retro-inhibition.* The allosteric activators are generally one of the substrates or cofactors of the enzyme. The effect of the allosteric "inhibitor" or 'activator' on the

enzyme has been found to be reversible. It means that if they are withdrawn, the enzyme regains the original activity.

An example of allosteric inhibition is the inhibition of threonine deaminase by isoleucine. Threonine deaminase deaminates threonine to form α-keto butyrate. However, the final product of the reaction is isoleucine. If there occurs the accumulation of isoleucine, conversion of threonine to α-ketobutyrate and therefore formation of other intermediates in the biosynthesis of isoleucine gets stopped. If isoleucine is consumed, threonine deaminase gets reactivated and reactions for the biosynthesis of isoleucine are started again.

An example of allosteric activation is activation of glycogen synthetase by glucose 6-phosphate. Another example of allosteric regulation (of both inhibitory and activating type) is seen in Pasteur effect. By *Pasteur effect* it means the inhibition of glycolysis and fermentation by oxygen. The molecular basis of this effect involves the allosteric inhibition of enzyme phosphofructokinase by ATP and citrate and its activation by AMP. This type of regulation is having adaptive significance. When the level of AMP increases due to increased use of ATP in the cell, glycolysis gets increased by the activation of phosphofructokinase. This results more formation of ATP. If ATP level becomes greater than the normal requirement of the cell, inhibition of glycolysis takes place through the same enzyme phosphofructokinase and ATP synthesis is stopped.

The mechanism of allosteric regulation assumes that there are two active centres in allosteric enzymes, one for the substrate and the other for effector. These two sites are present either on same or on two different subunits. When an effector molecule is binded to one type of subunit, the structure of the enzyme molecule is changed in such a way that binding of the substrate to the other subunit gets affected.

In order to explain the mechanism, an example of allosteric regulation of aspartate transcarbamylase may be considered.

In aspartate transcarbamylase there are two types of subunits. It is possible to split apart these two types of subunits by treatment with mercurials, when one type is retaining the ability to bind with the substrate, whereas the other is retaining the ability to recognize the inhibitor. If these two species of subunits are together (active enzyme molecule), binding of the inhibitor (CTP) to one type of subunit has been found to change the structure of other subunits in such a way that the binding of the substrate gets inhibited. If the subunits having binding sites for the inhibitor are removed, enzyme is not influenced by CTP. Further, a typical Michaelis-Menten curve is obtained with the substrate concentration. Similarly, the binding of activator may change the molecular structure in such a way that the binding of substrate gets facilitated.

2. Isozyme formation : Another phenomenon controlling cellular metabolism involves the formation of isozymes (isoenzymes).

Isozymes are different physical forms of the same enzyme which carry out the same general function at different rates. As they have been found to be different to some extent in their amino acid composition, they may be separated by involving electrophoresis. An important example is lactic dehydrogenase which catalyses the oxidation of lactate to pyruvate with the help of NAD^+.

$$CH_3CHOHCOOH \underset{NAD^+ \; NADH}{\overset{\text{Latic dehydrogenase}}{\rightleftharpoons}} CH_3COCOOH$$

The enzyme is tetramer having two distinct types (H and M types) of subunits. Depending upon the relative amounts of two types of subunits, lactate dehydrogenase has been found to form 5 isozymes as follows :

LD_1 = HHHH
LD_2 = HHHM
LD_3 = HHMM
LD_4 = HMMM
LD_5 = MMMM

The molecular weight of the enzyme has been found to be 135,000. If it is made to treat with urea of guanidine hydrochloride, it gets dissociated into subunits, each is having a m.w. of 35,000. The regulation of different isozymes has been found to be different. LD_1 (HHHH) type of lactate dehydrogenase occurs in the heart muscles. This species becomes most active at low pyruvate concentration and gets inhibited by high concentrations of pyruvate. LD_5 (MMMM) type of enzyme occurs in skeletal muscle cells and it has been found to remain active at high pyruvate concentrations.

Another example of isozyme formation is aspartokinase. It has been found to catalyse the reaction between aspartic acid and ATP when aspartyl phosphate is obtained. Amino acids lysine, methionine and threonine are final products of the reaction. The enzyme aspartokinase has been found to exist in three forms, namely, aspartokinase I, aspartokinase II and aspartokinase III. Aspartokinase, I gets inhibited by threonine and III by lysine. Aspartokinase II has been found to be insensitive to any of these amino acids. Thus, if any one of these amino acids gets accumulated, the synthesis of the other is affected very little.

3. Multienzyme system : There are some enzymes which do not exist as individuals but as aggregates of several enzymes and coenzymes. These aggregates are able to channel the metabolities in a pathway efficiently. In an aggregate each component is arranged in a way that the product of one enzyme becomes the substrate for the other and so on.

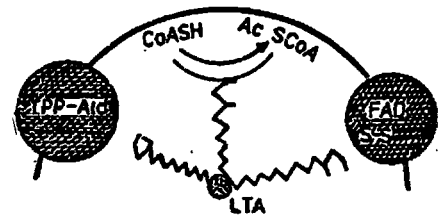


Fig. 27.1. A schematic representation of pyruvate dehydrogenase complex system.

Pyruvic acid dehydrogenase of *E. coli* is an example of enzyme aggregation. This complex is having three enzymes, pyruvate decarboxylase, dihydrolipoic dehydrogenase and lipoyl reductase trans-acetylase. The coenzymes which are associated with the complex have been found to be thiamine pyrophosphate (TPP) and flavin adenine dinucleotide (FAD). Pyruvate dehydrogenase complex is shown in Fig. 27.1. The stepwise reactions catalyzed by this complex may be put as follows :

$$\text{Pyruvate} + \text{thiamine pyrophosphate} \longrightarrow \alpha\text{-Hydroxyethyl thiamine pyrophosphate} + CO_2$$

$$\alpha\text{-Hydroxyethyl thiamine} + \text{Lipoate} \longrightarrow \text{Thiamine pyrophosphate} + \text{acetyl dihydrolipoate}$$

$$\text{Acetyl dihydrolipoate} + \text{CoASH} \longrightarrow \text{Acetyl CoA} + \text{dihydrolipoate}$$

$$\text{Dihydrolipoate} + NAD^+/FAD^+ \longrightarrow \text{Lipoate} + NADH_2/FADH_2$$

4. Regulation by adenylate energy change : The adenosine phosphates find importance in metabolic processes of living systems. The adenylate energy change may be regarded as the measure of total pool of adenosine phosphates in the form of ATP, ADP and AMP. It was D.E. Atkinson (1969) who defined adenylate energy change as follows :

$$\text{Adenylate energy change} = \frac{(ATP) + \frac{1}{2}(ADP)}{(AMP) + (ADP) + (ATP)}$$

In many systems, an increase in adenylate energy change in the physiological range brings about stimulation of regulatory enzymes. This is a well known phenomenon found in animals and micro-organisms. Further, it is also found in plants. Recently, Knotz *et al.* (1987) showed that the adenylate energy change has been found to effect the activity of pyrophosphomevalonate decarboxylase, the key enzyme which takes part in the biosynthesis of kaurene from mevalonate. An increase in enzyme activity has been found to be observed between adenylate energy change of 0.8 and 1.0.

Enzyme Induction and Enzyme Repression

In bacteria and certain other microbes, there occurs the synthesis of enzymes according to the need of the organism. In some higher plants and animals it has also been observed. For example, the enzyme nitrate reductase in the plants appears only if the substrate (NO_3^-) is supplied. In this there occurs *denovo* synthesis of the enzyme. The reason is that in the presence of protein inhibitor, cycloheximide, the enzyme activity remains low even if plenty of nitrate is available. Ammonium salts, and many amino acids have been reported to inhibit this substrate induction of nitrate reductase, especially in bacteria and fungi. In maize roots, it has been found that the inhibition of nitrate reductase by an amino acid analogue, canavanine, takes place the through repression.

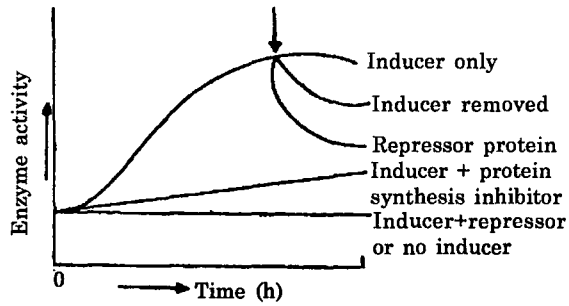


Fig. 27.2. Time kinetics of enzyme activity in response to inducer and repressor.

Genes have been reported to be governing induction and repression of enzymes. The process of induction of repression of enzymes has been found to be a much slower method of metabolic regulation than that of the allosteric or other kind of enzyme manipulation. The process needs several minutes to attain effectiveness. This lag period is the time required in expressing different genes in the operon. The inductor or repressor becomes effective only when it undergoes combination with the product of the regulator gene. In Fig. 27.2 there is a hypothetical graph which shows time kinetics of enzyme activity in response to inducer and repressor (Fig. 27.2).

There are many hereditary metabolic disorders of human beings which occur due to enzyme inductions and repression. It has been found that the altered activities of certain genes may result a change in the production of some key enzymes and thus the level of metabolites. If the operator part of a gene cluster (operon) gets inactivated by mutation of some other process, the products of structural gene(s) continue to be accumulating irrespective of the requirement of the cell.

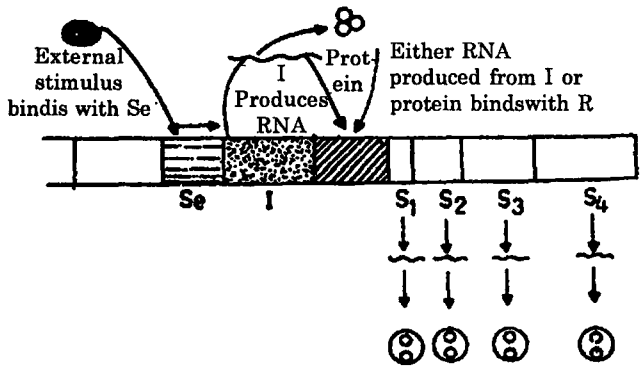


Fig. 27.3. Diagrammatic representation of the gene regulation in higher organisms (eukaryotes). Se is Sensor gene, I Integrator gene, R Receptor gene and S_1–S_4 Producer genes.

The operon model has been found to be suitable in explaining some isolated cases of regulation of synthesis of enzymes and other proteins. However, this model could not explain many other observations in higher organisms. Some of these observations are as follows :

(i) In the cells of higher organisms, the amount of DNA is high.

(ii) In higher organisms, there occurs repetition of the DNA sequences more frequently.

(iii) Accordingly to the operon model, the genes controlling related functions should be grouped together. However, they are not grouped together.

In order to explain the above observations, R.J. Britten and EH. Davidson (1969) postulated their own theory. According to them, there are at least five types of genes according to their function (Fig. 27.3).

1. Producer genes : These genes have been found to be analogous to the structural genes of the operon model. Producer genes are transcribed for producing *m*-RNA which will code for protein.

2. Receptor genes : Adjacent to producer genes there are receptor genes which work as a receptor for initiating transcription of producer genes. The molecules initiating transcription must bind with the receptor genes. Such molecules may be either a small RNA (termed as activator RNA) or a protein which is coded by that RNA.

3. Integrator genes : These genes have been found to synthesize activator RNA.

4. Sensor genes : These are the genes with receive the external stimulus for the activation of genes either in the form of a hormone or some other molecule. Generally the sensor genes have been associated with the integrator genes and hence if a stimulus gets attached with the sensor genes, the activity of adjacent integrator genes gets stimulated.

5. Battery of genes : It is a group of producer genes, which get activated when the corresponding group of integrator genes get activated by its sensor gene.

27.7 Compartmentation of the Metabolites

There are numerous specialised compartments in a cell called specialised cell compartments. Each of these compartments carries out a specific metabolic function. In a cell there are bigger compartments such as vacuoles, mitochondria and chloroplasts. In a cell there are also smaller compartments such as glyoxysomes, peroxysomes, lysomes and golgi bodies. These compartments are segregated from others by membranes and have all the essential enzymes for performing the specific function. This serves as control on the metabolism of organism. Hence synthesis of fatty acids taking place on endoplasmic reticulum gets separated from its degradation (in glyoxysomes and mitochondria). Golgi bodies are involved in cell wall synthesis and lysosomes are able to produce only some destructive hydrolytic enzymes.

Compartmentation in a cell has been found to regulate biochemical reactions in many ways :

(i) As substrates and enzymes are present in different compartments, this separation may inhibit some of the reactions. They will only mix up together only when it is required. The mixing up takes place either by changed permeability of the membranes isolating them or by activated diffusion. The same metabolite may also be distributed among different compartments.

(ii) In the cytoplasm, there are two pools of amino acids, *i.e.*, the storage pool and metabolic pool. The amino acids derived from the degradation of proteins get accumulated in the storage pool, while those destined to be incorporated into new proteins are accumulated in metabolic pool. The amino acids of the storage pool get largely catabolised to yield other carbon compounds and CO_2.

In the plant cells, there are two pools for a simpler compound called nitrate. An interesting example of nitrate compartmentation can be seen in maize seeding. Nitrate reductase gets induced in nitrate supply in all parts of the seedling including endosperm and scutellum. The only known function of the nitrate reductase is to bring about reduction of nitrate. There occurs no assimilation of nitrate, in the young seedlings though there exists an appreciable level of enzyme activity. Therefore, it is evident that nitrate in the young seedling gets accumulated largely in the storage pool, where it may act as an inducer of nitrate reductase but not to be assimilated by itself.

28 Metabolism of Inorganic Substances (MINERAL METABOLISM)

28.1 Introduction

In a normal diet, the mineral elements present in the animal body are present. In poor diets, there occur deficiencies of calcium and iron. This happens especially in underdeveloped countries. In hilly areas, there occurs deficiency of iodine in diets. In tropical countries, there occur excess loss of sodium chloride and therefore addition of sodium chloride in the diet is of vital importance. However, the deficiencies of other minerals do not take place normally in average diets.

The metabolism of certain minerals such as sodium and potassium is intimately connected with the water balance, osmotic pressure and pH regulation of the body and body fluids.

The mineral elements may be classified as *principal elements (macronutrients)* and *trace elements.*

Principal Mineral Elements (Macronutrients) : There are seven essential elements : Calcium, Magnesium, Sodium, Potassium, Phosphorous, Sulphur and Chlorine.

1. Sodium, potassium and chlorine are involved mainly in the maintenance of acid-base balance and osmotic control of water metabolism.

2. Calcium, phosphorous and magnesium act as constituents of bones and teeth.

3. Phosphorous forms a constituent of body cells of soft tissues, such as muscles, liver etc.

4. Sulphur forms a constituent in cysteine and methionine, thiamine, biotin, lipoic acid and CoA.

Trace Elements

These elements are found in living tissues in small amounts. They are subdivided into three groups—essential, possibly essential and non-essential.

1. Essential trace elements (micronutrients) : Iron, iodine, copper, zinc, manganese, cobalt, molybdenum, selenium, chromium and fluorine.

2. Possibly essential trace elements : Nickel, tin, vanadium and silicon.

3. Nonessential trace elements : Aluminium, boron, germanium, cadmium, arsenic, lead and mercury.

Physiological Importance

(i) Iodine is needed for thyroxine formation.

(ii) Iron and copper are needed for hemoglobin formation.

(iii) Zinc is a constituent of carbonic anhydrase and insulin.

(iv) Cobalt is a constituent of vitamin B_{12}.

The ratio of one element to the other in the tissues is of physiological importance, *e.g.,* normal ossification needs a proper ratio of calcium to phosphorous.

28.2 Calcium

Quantitatively calcium is present in largest amount because of its being the main mineral constituent of bone and teeth. Small amounts are also found in blood and body fluids and exert a regulatory function (depressent effect) on neuromuscular irritability and blood coagulation and also in the permeability of cell membranes and capillaries. The metabolism of this mineral is regulated by vitamin D and parathormone.

Sources

Richest Sources : Milk and cheese.

Good Sources : Egg yolk, nuts, figs, beans, cabbage cauliflowers, turnip greens and asparagus.

Daily Requirements

Adult males and females	800 mg
Women during pregnancy and lactation	1200 mg
Infants under 1 year	360-540 mg
Children 1-18 years	800-1200 g

Physiological Functions

1. Calcium along with phosphorus been found to be essential for the formation and development of bones and teeth.

2. Ionized calcium is needed in blood coagulation process.

3. It regulates the excitability of nerve fibres and nerve centres.

4. It is essential for nerve impulses and muscular contraction.

5. It tends to regulate the permeability of membranes.

6. It is essential for maintaining the integrity of intracellular material.

7. Calcium in the normal ratio with potassium is known to maintain the normal activity of muscles.

8. It is needed for the activation of several enzymes like succinate dehydrogenase, ATPase and certain proteolytic enzymes.

Absorption : Unlike sodium and potassium which are readily absorbed from the intestine, the absorption of calcium is incomplete. A high protein diet favours its absorption while a high cereal diet will diminish it. On a high protein diet, 15% of dietary calcium gets absorbed. If the protein content is low, only 5% might be absorbed. The cereals contain phytic acid (inositol hexaphosphate) which forms an insoluble salt with calcium. Oxalates, present in vegetables like cabbage and spinach, also prevent its absorption. An acidic pH of the intestine tends to favour absorption. A ratio of food calcium to phosphorous not more than 2 : 1 and not less than 1 : 2 is essential for optimal absorption of calcium. Vitamin D is able to promote its absorption. Presence of large amounts of unabsorbed fatty acids, as in sprue syndrome, will bring about its excretion as calcium soap.

Two mechanisms are used for the absorption of calcium by the intestinal mucosal cells.

1. An active transport process which is involving metabolic energy and a Ca^{2+} pump.
2. Simple diffusion.

Both the processes need 1, 25-dihydroxycholecalciferol. It regulates the synthesis of the proteins involved in Ca^{2+} dependent ATPase.

Glucocorticoids inhibit Ca^{2+} absorption by some, as yet unknown mechanism.

Plasma is having 9.0-11.0 mg/100 ml (5 m.eq/litre) of which about a half is in an ionizable and diffusible form and another half is protein-bound and non-diffusible. Small amounts also occur as citrate which is non-ionizable but diffusible. Erythrocytes do not have any calcium. The C.S.F. contains about 4.0-5.0 mg/100 ml. (2 m eq/litre) of calcium, especially in the ionizable form.

The effect of calcium of neuromuscular excitability (depressor effect) and on coagulation of blood are on account of the ionizable fraction.

The calcium in the glomerular filtrate is almost completely reabsorbed by the tubule. Only small amounts are present in urine (about 200 mg in 24 hours).

In the proximal convoluted tubule, calcium absorption has been associated with sodium reabsorption. In the distal tubule calcium is actively transported unrelated to sodium reabsorption. Parathyroid hormone is known to stimulate this active reabsorption by the distal convoluted tubule.

Blood Calcium

There is almost no calcium in erythrocytes. The calcium content of plasma (usually determined in serum) is 9 to 11 mg/100 ml. During infancy and early childhood, the average values approach the upper limit of this range and decrease with advancing age. Calcium is found in the plasma in three fractions ionized or diffusible calcium, protein bound (nondiffusible) and complexed (citrate and phosphate). In the usual determination of calcium, all these fractions are measured together.

About 2 mg of the total calcium is found in ionized form, about 5 mg is found in non-ionized form and about 2 mg in the complex form.

Factors Influencing Blood Calcium Level

1. Parathyroid hormone : In the fasting state (*i.e.,* no absorption from the intestine), the normal plasma calcium concentration is maintained primarily by its rate of excretion and its mobilization from the bones through the action of the parathyroid hormone.

2. Vitamin D : It increases absorption of calcium from the intestine and thus maintains the normal plasma calcium concentration.

3. Plasma proteins : Half of the blood calcium (nondiffusible fraction) is bound to plasma protein (chiefly albumin) and thus any decrease in these proteins will be accompanied by a decrease in the total calcium level.

4. Plasma phosphate : A reciprocal relationship exists between the concentration of calcium and phosphate ions in plasma. The marked increase in serum phosphate brings about a fall in serum calcium concentration.

5. Calcitonin : An increase in the ionized calcium levels in the plasma is the stimulus for the production of calcitonin which then brings about a deposition of calcium in bones.

Excretion

Calcium gets excreted in the urine, bile, and digestive secretions. Much of that is excreted in the feces which has escaped absorption. Under optimal conditions, 75 per cent of dietary calcium gets absorbed. The remainder is the fecal calcium which gets unabsorbed.

In man, about 10 grams of calcium get filtered in 24 hours by the renal glomeruli. Only about 200 mg appear in the urine which is in the ionic state as well as in the complexes with citrate and other organic anions.

70-90 per cent of the calcium eliminated from the body is excreted in the feces.

A very small amount gets excreted into the intestine after absorption.

The daily loss of calcium in sweat is about 15 mg. Vigorous physical exercise increases the loss of calcium by the way of sweat.

Excretion of calcium occurs mainly through feces and reflects the unabsorbed portion of dietary calcium.

Abnormalities in calcium metabolism : Parathormone brings about alterations mainly in the diffusible portion of calcium in plasma.

(i) Hyperparathyroidism : In this condition the plasma calcium levels will reach upto 12-20 mg/100 ml. There is a corresponding decrease in phosphate level. The increase of calcium in plasma is mainly by demineralization of bone leading to entry of calcium and phosphorus from bone into blood.

By diminishing tubular reabsorption of phosphate in the kidney, the parathormone causes an increase in its urinary loss and lowers serum phosphate levels.

There also occurs increased excretion of calcium in urine.

(ii) Hypoparathyroidism : In this condition, the serum calcium levels (particularly the ionized and diffusible fractions) are reduced to 7 mg/100 ml or below and the serum phosphate levels increase due to a decreased excretion in urine. A condition called 'tetany' occurs which is characterized by increased neuromuscular irritability, spasms and convulsions.

(iii) Rickets : Apart from a deficiency of vitamin D, a deficit in calcium and phosphorous in the diets of children can give rise to the development of rickets. In children, serum inorganic phosphate levels vary from 4-7 mg per 100 ml. In rickets, it may fall to as low as 1-2 mg%.

(iv) Relationship to plasma phosphate : There is a sort of an inverse relationship with plasma phosphate levels. The plasma Ca × P product is $10 \times 4 = 40$ in a normal adult.

The Ca × P product in children is normally 50. It gets lowered to 30 in rickets.

(v) Renal rickets : Here the primary defect lies in the absorption of calcium and phosphorus from the intestinal epithelium and the reabsorption of phosphate by the renal tubule. There occurs a gross increase in the urinary excretion of phosphate (hyperphosphaturia) and lowered blood calcium and phosphate levels. The condition is not relieved by vitamin D and is hence called 'vitamin D resistant rickets.' It is a familiar disease which is transmitted as an X-linked dominant trait, affecting male children.

(vi) Relationship to plasma proteins : As about 5 mg/100 ml of plasma calcium is protein-bound, mainly to the albumin fraction, any decrease in plasma proteins (hypoproteinemia) is associated with a decrease in the non-diffusible, protein-bound fraction of calcium. The role of calcium in bone formation has been discussed under vitamin D.

28.3 Phosphorus

Like calcium, it forms a predominant constituent of bone and teeth. But it is also found in every cell of the body in combination with proteins, lipids and carbohydrates (*e.g.*, phosphoproteins, phospholipids and hexose phosphates). As a constituent of ATP and similar compounds, it plays a unique role in energy storage and transformations.

Requirement

Nearly 800 mg are needed per day for an adult. It is found together with calcium in most of the sources mentioned for calcium. Proteins of food which do not have calcium also supply good amounts of phosphorous. Blood plasma has 3-5 mg/100 ml. as inorganic phosphate (1.0-1.5 mM/ litre). Total blood phosphorus is 40 mg/100 ml.

Daily Requirements

Infants	240-400 mg
Children	800-1200 mg
Adults	800 mg
Women during pregnancy and lactation	1200 g

Physiological Functions

1. It is essential for the formation and development of bones and teeth along with calcium.
2. It is needed for the formation of phospholipids, nucleic acids, and phosphoproteins.
3. It gets involved in the formation of organic phosphates, such as hexose phosphate, triose phosphate and creatine phosphate etc.
4. It is needed for the formation of energy rich compounds, such as ATP.
5. It forms coenzymes, such as NADP, ADP, AMP and B_6 - PO_4 etc.
6. It functions in the buffering system in cells.
7. It is needed in the absorption of glucose of phosphorylation.

Distribution

Serum (inorganic phosphorus) of

(a)	Children	4-7 mg/100 ml
(b)	Adults	3-4.5 mg/100 ml
	Muscle	170-250 mg/100 g
	Nerve	360 mg/100 g
	Bones and teeth	22,000 mg/100 g

Absorption

1. Moderate amounts of fat or acid tend to favour absorption of phosphorus.
2. High calcium diet and phytic acid (present in cereals) causes a decrease in phosphorus absorption.
3. The absorption gets enhanced when the calcium and phosphorus ratio becomes 1:1 (2:1 or 1:2).

Blood Phosphorus

The normal inorganic phosphate of plasma is 3 to 4.5 mg/100 ml in adults and is 4.5 to 6.5 mg/100 ml. in children. It is somewhat higher in summer than in winter. It decreases during increased carbohydrate metabolism due to increased utilization for phosphorylation.

Phosphorus occurs in the blood in the following forms :

1. Inorganic phosphorus	2-5 mg/100 ml
2. Organic phosphorus	14-29 mg/100 ml
3. Phospholipids	8-18 mg/100 ml

Excretion

Inorganic phosphorus gets excreted in the urine and feces. The source of urinary inorganic phosphorus is mainly that of plasma. On a balanced diet, urine phosphate constitutes about 60 per cent of the total excretion. The rest gets excreted in the feces. The "rental threshold" for phosphate excretion is about 2 mg/100 ml. of plasma. The reabsorption of phosphorus gets inhibited by the parathyroid hormone.

Disease State

1. In rickets, serum phosphate level becomes as low as 1–2 mg/100 ml.

2. A temporary decrease in serum phosphate takes place during absorption of carbohydrate and some fats.

3. A lower concentration of organic phosphorus but a higher concentration of inorganic phosphorus in the serum has been estimated in diabetes mellitus.

4. Phosphate retention brings about the acidosis in severe rental disease. The resultant is the increase in serum phosphorus level.

5. Serum phosphorus levels get increased in hypoparathyroidism.

6. Blood phosphorus levels get decreased in hyperparathyroidism and in celiac disease.

7. In renal rickets there occurs low blood phosphorus level with an increased alkaline phosphatase activity.

8. The deficiency of vitamin D is considered to be the cause of the low serum phosphorus and the defects in the calcification of bones.

28.4 Magnesium

Seventy per cent of body magnesium is found in bones in association with calcium and phosphorus. The rest is found in all the cells of the other tissues and in blood and body fluids. Blood is having 2-4 mg/100 ml (1.7 to 3.4 m.eq/litre). It is about equally distributed between cells and plasma. C.S.F. is also having about 3 mg/100 ml.

Sources : It is present in milk, eggs, cabbage, cauliflowers and fruits etc.

Distribution

Whole blood	2-4 mg/100 ml
C.S.F.	3 mg/100 ml
Muscle	21 mg/100 gm

Daily Requirement

Infants	100-150 mg
Children	150-200 mg
Adults	200-300 mg

Blood Magnesium

The normal level of magnesium in blood is 1–3 mg/100 ml.

Physiological Functions

It is necessary for the activity of many enzymes in carbohydrate metabolism (*e.g.,* hexokinase, phosphofructokinase, phosphoglyceratekinase, enolase, pyruvate kinase, adenyl cyclase, phosphoglucomutase, pyruvate dehydrogenase, glucose-6-phosphate dehydrogenase, transketolase) and others; (in some of these, calcium or manganese may replace Mg^{2+}). Together with calcium and hydrogen ions, it is able to depress neuro-muscular activity and balances the action of Na^+ and K^+.

2. Magnesium ions act as activators for many of the phosphate group transfer enzymes.

Sources : 300-350 mg/day are needed for adult. Nuts, beans, cereals, vegetables, meats and milk supply adequate amounts in a normal diet. High amounts of calcium and phosphorus in diets and ingestion of large amounts of alcohol will decrease magnesium absorption. Normally about 50% of dietary magnesium gets absorbed.

Absorption

1. A greater part (40 to 50 per cent) of the daily ingested magnesium (200 to 300 mg) is not absorbed.

2. Very high intake of fat, phosphate, calcium and alkalis diminish its absorption.

3. Parathyroid hormone tends to increase its absorption.

Excretion

This occurs mainly through feces (unabsorbed dietary magnesium). Absorbed magnesium is excreted through urine, bile and intestinal secretions. Increase in plasma levels of magnesium depresses the nervous system and if sufficiently high is able to induce anaesthesia and paralysis of skeletal muscle. Magnesium and potassium are normal cations of the intracellular fluid. Aldosterone brings about an increased urinary excretion of both potassium and magnesium.

Disease State

1. Magnesium deficiency brings about depression, muscular weakness, and liability to convulsions. The serum magnesium level is below 1 mg/100 ml.

2. Its deficiency can also be observed in chronic alcoholics with the low serum magnesium and muscular weakness.

3. In cases of kwashiorkor, the serum magnesium level is low causing weakness.

4. Low values for serum magnesium have been found to be in uremia, normal and abnormal pregnancy, rickets, growth hormone treatment, hypercalcemia and the recovery phase of diabetic coma.

28.5 Sodium

In the excellular fluids, sodium is the chief cation and chloride an important anion whereas in the intracellular fluids, potassium is the chief cation. Sodium usually enters the cell but is rapidly removed by what is known as sodium pump, an energy requiring mechanism of transport from within the cell to extracellular fluid against gradient.

Sources : The *main source* of sodium is the sodium chloride used in cooking and seasoning.

Rich sources : Bread, cheese, wheat germ, whole grains and oysters etc.

Good sources : Carrots, cauliflowers, eggs, milk, nuts, spinach and turnips etc.

Distribution : About one-third of the total sodium content of the body is present in the inorganic portion of the skeleton. Most of the sodium is found in the extracellular fluid.

Plasma	330 mg/100 ml
Cells	85 mg/100 gm
Muscle	60-160 mg/100 gm
Nerve	312 mg/100 gm

Daily Requirement

For adults the daily requirement is 5 to 15g. In temperate region, the sodium chloride intake is less but in tropical countries, the intake is more. A person suffering from hypertension should not take more than 1 g of sodium.

Physiological Functions

1. It is found to be the major component of the cations of the extra-cellular fluid. It exists in the body in association with the anions such as chloride, bicarbonate, phosphate and lactate.

2. It is largely associated with chloride and bicarbonate in regulation of acid-base equilibrium.

3. It helps to maintain the osmotic pressure of the body fluid and thus protects the body against excessive fluid loss.

4. Sodium ion plays a vital role in the absorption of glucose and galactose as well as amino acids from the small intestine.

5. It maintains the normal water balances and distribution.

6. Sodium ion gets involved in initiating and maintaining the heart beat.

7. It maintains the normal neuromuscular function.

8. It functions in the permeability of the cells.

Absorption : Normally, sodium is almost completely absorbed from the gastrointestinal tract. Less than 2 per cent of ingested sodium gets eliminated in the feces. In subjects with diarrhea, large amounts get lost in the feces.

Blood sodium : The normal level of sodium is 310-340 mg/100 ml. In man, erythrocytes is having little or no sodium. Aldosterone increases plasma sodium level.

Excretion : The daily losses of sodium are as follows :

Urine	5-35 mg
Stool	10-125 mg
Skin (not sweating)	25-25 mg
Total	40-185 mg

Nearly 95 per cent of the sodium leaving the body gets excreted in the urine because sodium is readily absorbed in the intestine. Therefore, feces contain very little sodium except in diarrhea. There occurs the variable loss of sodium by way of the sweat. Heavy exercise, environmental heat and high fever can cause excessive losses of sweat which leads to more sodium losses. Loss of sodium by excessive sweating brings about *heat cramps* with the intense and painful contractions of skeletal muscle of men working hard in hot humid climates.

Disease State

1. Sodium deficit : There are occur loss of the extracellular fluid (ECF) sodium with or without simultaneous fluid loss. This may happen in severe burns, peritonitis, large ascites and pleural

effusion and severe edema. The loss of sodium in these cases is also accompanied by loss of plasma albumin. Sodium loss can also take place in severe vomiting or diarrhoea. Administration of cation-exchange resins may also cause it. Excessive sweating may give rise to substantial losses of sodium through the skin. If lot of plain water is ingested to allay thirst, the resulting dilution of plasma magnifies the sodium deficit even more, and when the kidneys remove the extra water, a further loss of sodium may occur through the urine.

Inability of the renal tubule to reabsorb normal amounts of sodium can take place in salt losing nephritis. Water retention because of excessive ADH action can also bring about dilution of plasma and low sodium levels. In Addison's disease, the plasma sodium levels are low. In conditions like diabetic acidosis, therapy with mercurial diuretics or acetazolamide, there occurs an increased urinary excretion of sodium.

The affected subject will show weakness, apathy, headache, giddiness, muscular cramps and in severe depletion, delirium and coma may occur. The eyeballs become shrunken and soft, tongue becomes shrunken and wrinkled, and skin becomes inelastic.

Packed cell volume (hematocrit) is usually increased and serum sodium is lowered. Serum potassium and urea get increased. Urine volume and particularly sodium content of urine are markedly decreased.

The condition can be treated by administering isotonic saline or plasma intravenously. In a few cases, hypertonic saline (3-5%) is indicated. Where the sodium deficit occurs due to loss of gastrointestinal secretions (vomiting, diarrhoea), there occurs likely to be a potassium deficit also. This has also to be made good.

2. Sodium excess : Serum sodium concentration does not reflect the total body sodium levels. Low serum sodium level can be associated with low, normal or high body sodium. Similarly, a high serum sodium may also get associated with any of the above three states of body sodium.

Increase in total body sodium is always associated with edema. It can occur due to circulatory failure, excessive production of excessive action of aldosterone and ADH.

The underlying cause of edema is to be treated.

3. In certain stages of pregnancy, the steroid hormones cause the retention of sodium as well as water which results in gain in weight.

28.6 Potassium

In the intracellular fluid, potassium is the chief cation. Potassium normally moves out of the cell only in small amounts. In severe dehydration of the cells, there may be a more rapid movement out of the cell.

Sodium and potassium on one hand and calcium, magnesium and hydrogen ion on the other hand maintain the normal neuromuscular irritability.

Irritability varies as : $\frac{Na^+ + K^+}{Ca^{++} + Mg^+ + H^+}$

About 4 grams are present in normal diets. Its deficiency is rare. Whole blood contains 200 mg/100 ml but plasma contains only 20 mg/100 ml. (5.m.eq/litre). It occurs in all tissues.

Sources : The high content of potassium is found in chicken, beef liver, bananas, the juices of oranges, pineapples, yams, winter squash and potatoes etc.

Distribution

Plasma	20	mg/100 ml
Cells	440	mg/100 gm
Muscles	250-400	mg/100 mg
Nerves	530	mg/100 mg

Daily Requirement

The normal intake of potassium in food is about 4 g. It is so widely distributed in food that its deficiency is rare except under pathological conditions.

Blood Potassium

The normal level of potassium in serum is 14–20 mg/100 ml. In man, erythrocytes are having large amounts of potassium. Therefore, care has to be taken to avoid hemolysis for the determination of serum potassium. The serum potassium decreases during increased carbohydrate utilization following administration of glucose or insulin.

Aldosterone lowers serum level.

Physiological Functions

1. Potassium is largely found in the intracellular fluid and it is also found in small amounts in the extracellular fluid because it influences cardiac muscle activity.

2. It plays an important role in the regulation of acid-base balance in the cell.

3. It is able to maintain osmotic pressure.

4. It functions in water retention.

5. It is essential for protein biosynthesis by ribosomes.

6. The glycolytic enzyme pyruvate kinase needs K^+ for maximal activity.

Absorption

Normally, potassium is almost completely absorbed from the gastro-intestinal tract and less than 10 per cent of potassium gets eliminated in the feces. In subjects with diarrhea, large amounts of potassium get lost in the feces.

Excretion

Potassium is normally eliminated almost entirely in the urine and a small amount in the feces. Aldosterone exerts an influence on potassium excretion. In the presence of normal kidney function, potassium is very efficiently and immediately removed from the blood.

Disease State

1. Potassium is not only filtered by the kidney but also secreted by the renal tubules. The excretion of potassium is greatly influenced by changes in acid-base balance and also by the activity of the adrenal cortex. The capacity of the kidney to excrete potassium is very great and therefore, hyperkalemia does not take place even after the ingestion of potassium if the kidney function gets unimpaired. Potassium should not be given intravenously unless circulatory collapse and dehydration are corrected.

2. *Hyperkalemia* takes place in patients in the following conditions : *(i)* Renal failure, *(ii)* Severe dehydration, *(iii)* Addison's disease due to decreased excretion of potassium by the kidney, *(iv)* Intravenous administration of excessive amounts of potassium salts, *(v)* Shock.

The *symptoms* of hyperkalemia have been cardiac and central nervous system depression. The heart signs include bradycardia and low heart sounds followed by peripheral vascular collapse leading to cardiac arrest. The other symptoms have been mental confusion, numbness, weakness of respiratory muscles and flaccid paralysis of the extremities. It is possible to correct the symptoms by administration of deoxycorticosterone which helps the excretion of potassium.

3. Prolonged *hypokalemia* brings about injury to myocardium and kidneys. Potassium deficits take place in chronic wasting diseases with malnutrition, prolonged negative nitrogen balance, gastrointestinal losses and in metabolic alkalosis.

The clinical conditions exhibiting hypokalemia may be as follows :

(i) Prolonged diarrhea and vomiting with the loss of digestive juices.

(ii) Intravenous administration of potassium-free fluid for replacing digestive juices lost by prolonged vomiting.

(iii) Overactivity of adrenal cortex (Cushing's syndrome) which brings about increased excretion of potassium in urine.

(iv) Prolonged use of diuretics.

(v) Heart failure treatment with digitalis.

(vi) Diabetic coma treatment with insulin.

(vii) In 'familial periodic paralysis', a rare disease, potassium is withdrawn from extracellular fluid and retained in the cells.

The *symptoms* of hypokalemia include muscular weakness, irritability, paralysis, tachycardia and dilatation of the heart with gallop rhythm and changes in the electrocardiogram (ECG).

28.7 Chlorine

It exists mainly as sodium chloride in blood and plays a vital role in the water balance and osmotic pressure and pH regulation. It is also necessary for the formation of HCl by the gastric mucosa.

It is taken as sodium chloride mainly and a deficiency or surplus of sodium and chloride occur together. The chloride content of cerebrospinal fluid is higher than that of plasma on account of the lower protein content. Chloride balances the anion content of C.S.F.

Distribution

Plasma	365 mg/100 ml
Cells	190 mg/100 gm
C.S.F.	440 mg/100 ml
Muscle	40 mg/100 gm
Nerve	171 mg/100 gm

Daily Requirement

The requirements of NaCl has been found to depend on the climate and occupation and on the salt content of the diet. Foods of animal origin have more NaCl than those of vegetable origin. The daily requirements in tropical countries are given as follows :

Adults	10-20 g
Children	5-10 g
Women during pregnancy and lactation	10-15 g

Excessive consumption of NaCl brings about edema in protein deficiency and increases blood pressure in hypertension patients.

Blood Cl : The normal level of Cl in serum is 96-105 mmol/liter. In man, erythrocytes are having smaller amounts of Cl^-. The distribution of Cl^- between plasma and erythrocytes is related to that of HCO_3^-. The Cl^- content of whole blood becomes relatively high in anemia and relatively low in polycythemia. The serum Cl^- may fall during active gastric secretion of HCl.

Physiological Functions

1. As a component of sodium chloride, chloride ion is necessary in acid-base equilibrium.
2. As chloride ion, it is also necessary in water balance and osmotic pressure regulation.
3. It is also important in the production of hydrochloric acid in the gastric juice.
4. Chloride ion is an important activator of amylase.

Absorption : Normally, Cl^- is almost completely absorbed from the gastrointestinal tract.

Excretion : Cl^- is chiefly eliminated in the urine. It also gets excreted in the sweat. It is lost more during excessive sweating in hot climates under hard work. Its concentration in sweat gets decreased by aldosterone.

Disease State

1. Chloride deficit takes place when losses of sodium become excessive in diarrhea, sweating and certain endocrine disturbances.

2. A loss of chloride may take place in the loss of gastric juice by vomiting or in pyloric or duodenal obstruction.

3. Hypochloremic alkalosis may develop in Cushing's disease or after the administration of ACTH or cortisone.

28.8 Sulphur

It is present in all cells in association with protein in the sulfur containing amino acids, cysteine and methionene. It is ingested in the form of protein mainly and is eliminated in the urine in three forms described as inorganic, organic and ethereal sulfates totalling about 0.5 to 1.0 gram a day. The formation of inorganic sulfate takes place mainly in the liver.

Cystine, methionine, glutathione, heparin, insulin, anterior pituitary hormone, thiamine, lipoic acid, biotin, coenzyme A, ergothionine, and taurocholic acid are some of the sulfur containing compounds.

Sources : Sulfur intake mainly occurs in the form of cystine and methionine present in proteins. Other compounds present in the diet contribute small amounts of sulfur.

Sulfur in Blood

The normal concentration of sulfur in the serum is given as follows :

Inorganic sulfur	0.5-1.1 mg/100 ml
Ethereal sulfate	0.1-1.0 mg/100 ml
Neutral sulfur	1.7-3.5 mg/100 ml

Physiological Functions

1. Sulfur occurs primarily in the cell protein in the form of cysteine and methionine. The cysteine is important in protein structure and in enzymic activity. Methionine is the principal methyl group donor in the body. The "activated" form of methionine, S-adenosylmethionine, is the precursor in the synthesis of large number of methylated compounds which are involved in intermediary metabolism and detoxification mechanism.

2. Sulfur is a constituent of coenzyme A and lipoic acid which are used in the synthesis of acetyl-CoA and S-acetyl lipoate respectively.

3. Sulfur is a constituent of other organic compounds, like as heparin, glutathione, thiamine, biotin, ergothioneine, taurocholic acid, sulfocyanides, indoxyl sulfate, chondroitin sulfate, insulin, penicillin, anterior pituitary hormones and melanin.

Absorption

Inorganic sulfate gets absorbed as such from the intestine into the portal circulation. A small amount of sulfide may be formed in the bowel by the action of bacteria, but, if absorbed into the blood stream, this rapidly gets oxidized to sulfate.

Excretion

Sulfur gets excreted in the urine in three forms. The total sulfate excretion may get decreased

in the presence of renal functional impairment and gets increased in conditions accompanied by excessive tissue protein breakdown, like high fever and increased metabolism.

Disease State

1. The serum sulfate concentration gets increased in the presence of renal functional impairment, pyloric and intestinal obstruction and leukemia.

2. Marked sulfate retention in advanced glomerulonephritis bring about the development of acidosis.

3. An increase in the blood indoxyl concentration (indoxyl potassium sulfate) may take place in uremia.

TRACE ELEMENTS

28.9 Iron

Iron is a component of hemoglobin, myoglobin and cytochromes. It plays a key role in oxygen transport and cellular oxidations. The total iron content of the normal adult is about 4 to 5 g. Nearly 60 to 70 per cent of the total iron is present in hemoglobin and only about 0.1 per cent is carried in the plasma in combination with the β-globulin transport protein transferrin. The hemoprotein and flavoprotein enzymes together make up less than 1.0 per cent of the total iron. Large amounts are found as hemosiderin.

Adult man needs about 10 mg. iron daily in his diet. Growing children and pregnant and lactating women need more. Even non-pregnant women need more because of the periodical loss of blood during menstruation. Organ-meats like liver, heart and kidney, egg yolk, green leafy vegetables, whole wheat and molasses are good sources.

Iron in the blood : The normal concentration of iron in blood is 75-175 mcg/100 ml.

Sources

Rich sources : Liver, heart, kidney, spleen.

Good sources : Egg yolk, fish, nuts, figs, dates, beans, spinach, molasses, apples, bananas etc.

Poor sources : Milk, wheat flour, polished rich and potatoes etc. Human milk contains 0.3 to 0.6 μg iron/ml.

Daily requirement : About 10 per cent of the ingested iron is only absorbed.

Infants	10-15 mg
Children (1 to 3 years of age)	15 mg
(4 to 10 years of age)	10 mg
Older children and adults (Males)	
(11 to 18 years of age)	18 mg
(after 19 years of age)	10 mg
Females :	
11-15 years of age and during pregnancy or lactation 18 mg	18 mg
After 51 years of age	10 mg

In adult women, the average loss of blood during a menstrual period is a monthyl loss of 16-32 mg. of iron or an additional average loss of 0.5-1.0 mg per day. This amount can be readily supplied by the diet.

In excessive menstrual blood loss and in chronic iron-deficiency anemia, a supplement of 100 mg of iron per day is sufficient to respond. So during growth, pregnancy and lactation, iron demand is somewhat more.

In healthy adult male or in healthy women after menopause, the dietary requirement is negligible unless any deficiency or loss of iron takes place.

The iron deficiency takes place as a result of malabsorption from the gastrointestinal tract. A defect in hemoglobin synthesis in anemia is commonly found in copper deficiency.

Physiological Functions

1. The main function of iron is in the transport of oxygen to the tissues (hemoglobin)
2. It is also involved in the processes of cellular respiration.
3. It is an essential constituent of hemoglobin, myoglobin, cytochromes and the respiratory enzyme systems (cytochrome oxidase, catalase and peroxidase),
4. The nonheme iron is completely protein-bound which is found in the form of storage and transport.
5. The non-heme iron is also used in the structure of xanthine dehydrogenase and succinate dehydrogenase and also in the iron-sulfur proteins of the respiratory chain.

Distribution

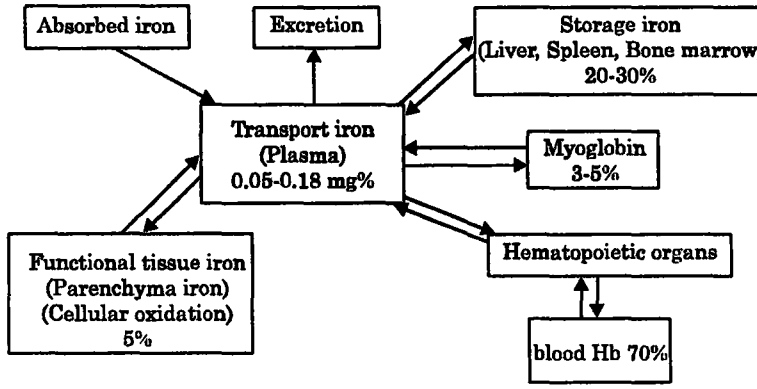


Fig. 28.1. Approximate distribution of iron in the body.

Iron content of some tissues

Tissue	*Total amount present*	*% of total body iron*
Hemoglobin	2500 mg	67
Storage (ferritin and hemosiderin)	1000 mg	27
Myoglobin	130 mg	3.5
Other tissues	88 mg	2.4
Transport iron	3 mg	0.1

Absorption

Under normal conditions, very little dietary iron gets absorbed (less than 10 per cent), the amounts excreted in the urine are minimal. Infants and children absorb a higher percentage of iron from foods than adults. Iron deficiency in infants is ascribed to a dietary deficiency. Iron-deficient children absorb twice as much as normal children.

Most of the iron in food exists in the ferric (Fe^{+++}) state either as $Fe(OH)_3$ or as ferric organic compounds. These compounds are broken down into free ferric ions or loosely bound organic iron.

The gastric hydrochloric acid and the organic acids of the foods are both important for this purpose. Reducing substances in foods, such as cysteine and ascorbic acid are able to convert ferric ion into the ferrous (Fe^{++}) state. In this form it is more soluble and therefore readily absorbed. Iron absorption gets increased by proteins of low molecular weight digestive products (peptides, amino acids) forming iron chelates. Heme enters the mucosal cells without being released from the porphyrin ring. In humans, dogs and rats, heme is broken down in the mucosa and iron appears in the plasma transferrin.

Factors Effecting Iron Absorption

1. Absorption of iron takes place mainly in the stomach and the duodenum. Impaired absorption occurs in patients who have total removal of stomach or a removal of the considerable amount of the intestine.

2. A diet high in phosphate brings about decreased absorption due to the formation of insoluble ferric phosphate ($FePO_4$). Very low phosphate favours increased absorption of iron.

3. Phytic acid (present in cereals) and oxalates are known to interfere absorption.

4. Vitamin C causes an increased absorption.

5. Gastric acidity increases absorption by converting $Fe(OH)_3$ to Fe^{3+}. Achlorhydria and administration of alkali decrease absorption.

6. Proteins of low molecular weight favour absorption.

7. Copper deficiency also causes decrease in absorption.

8. Alcohol ingestion favours iron absorption.

Mechanism of Absorption

Previously, "Mucosal Block" theory was regarded to control iron absorption. The iron-binding protein, *apoferritin,* in the mucosal cells, was the controlling factor. Ferrous ion being oxidized to ferric ion reacts with apoferritin to form iron containing protein.

For some time it was believed that the absorption depended on the formation of ferritin. When apoferritin got saturated with iron, no further uptake of iron could take place.

More recently, evidences reveal that ferritin gets involved in the major regulation of iron absorption. Iron taken into the mucosal cell is bound to specific carriers which regulate its passage across the cell to the blood. Intestinal ferritin, therefore, acts as a storage compound rather than the controlling of absorption.

Transport in the Plasma

All the iron released from the mucosal cell enters the portal blood in the ferrous state. In the plasma, ferrous oxidized to ferric state by ceruloplasmin (a copper binding plasma protein) exerts a catalytic activity (serum ferroxidase) in plasma. Human serum has also a yellow cuproprotein (ferroxidase II) which catalyzes the oxidation of ferrous ions. Ferric ion is then incorporated into a specific iron binding protein, *transferrin* or *siderophili,* which is a glycoprotein of molecular weight 76,000 containing 5.3 per cent carbohydrate. Transferrin can bind 2 atoms of ferric ions per molecule of protein to form a red ferric-protein complex. Iron release from the mucosal cell gets facilitated by a low degree of transferrin saturation by iron.

Under normal circumstances, nearly all the iron bound to transferrin is taken up readily by bone-marrow. Only the reticulocytes can use the ferric ion bound to transferrin, although reticulocytes and the mature erythrocytes can take up unbound ferric ion. The iron with transferrin, makes a complex which is not filtrable by the kidney. The total iron with transferrin,

makes a complex which is not filtrable by the kidney. The total iron binding capacity in both sexes is 330-360 μg/dl.

Losses of iron into the urine take place in proteinuria. In nephrosis, iron (1.5 mg/day) with protein may be excreted in the urine. In hepatic disease, both the bound iron and the total iron-binding capacity of the plasma become low.

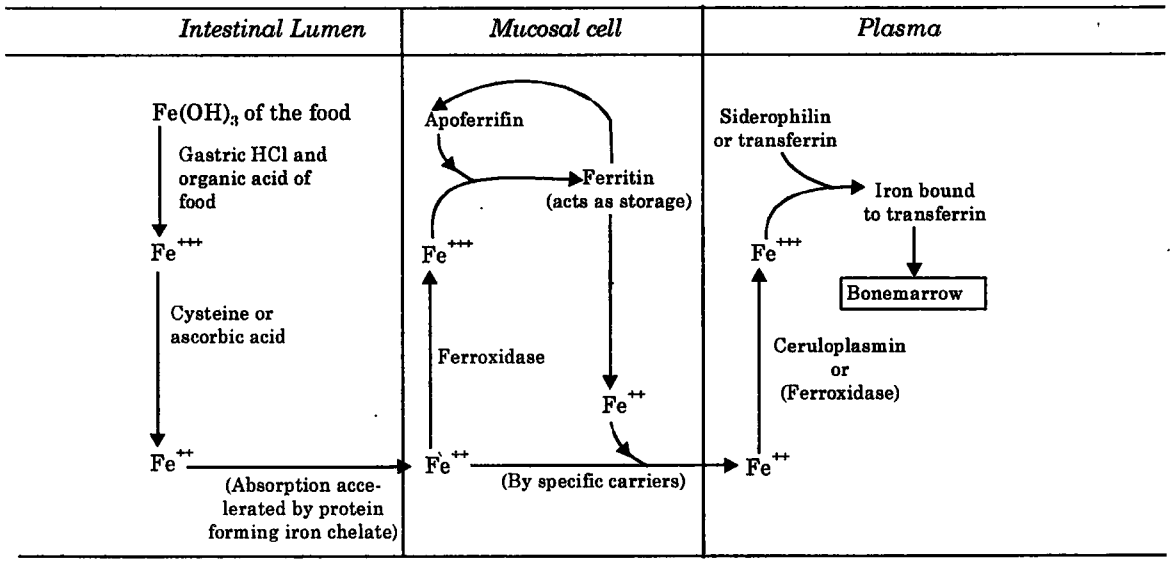


Fig. 28.2. Iron absorption and transport mechanism.

Excretion

The body stores of iron are conserved very efficiently. Only minute amounts are excreted in the urine, feces and sweats. Relatively large amounts get lost in the menstrual flow. The bulk of the iron of the feces is unabsorbed food iron. In the tropics, iron loss is often much greater. During pregnancy, iron is lost to the fetus. Iron is also lost from the skin by means of sweat, hair loss and nail clippings.

The daily excretion of iron may be as follows :

Adult male	0.5–1.5 mg in the urine
Women during menstruation double the above in the urine	
Adult (Both Male & Female)	0.5–1.0 mg in the sweat
Adult (Both Male & Female)	0.3-0.75 mg in the feces.

Storage of Iron : The liver, spleen and bone marrow all have apoferritin which can store iron by being converted to ferritin. The passage of iron from transferrin to apoferritin follows similar steps as earlier. The iron gets released as ferric iron from transferrin, reduced to ferrous form, taken up by the cells of the concerned tissues, reoxidized to ferric form and incorporated into apoferritin to form ferritin. If the iron supply is far in excess of demand (usually due to a breakdown in the mucosal block or due to parenteral administration), iron is stored in some of these viscera in a different form as 'hemosiderin' which is also a protein containing 35% iron. Hemosiderin becomes visible under the micro scope with suitable staining, whereas ferritin is not.

Utilization of Iron : The daily synthesis of hemoglobin needs about 27 mg a day. That much is liberated every day from the breakdown of hemoglobin and is almost completely reutilized. Thus very small amounts only are required from absorbed iron, unless there is a loss of blood as in hemorrhage. Iron is also needed for the activity of certain enzymes, *e.g.*, aconitase.

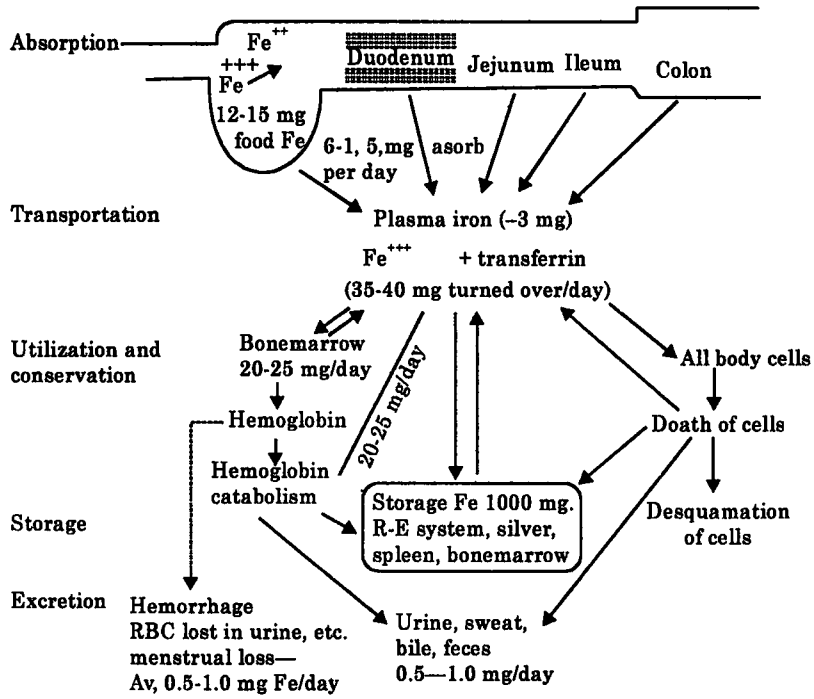


Fig. 28.3. Schematic representation of iron metabolism in man.
RBC = Red blood cells. RE system = Reticuloendothelial system.

Abnormal Iron Metabolism

Ferritin and hemosiderin are the storage forms of iron. These act as an internal iron reserve to protect against sudden losses of iron by bleeding. *Ferritin* occurs not only in the intestine but also in liver (about 700 mg.), spleen and bonemarrow. If more iron is administered parenterally exceeding the capacity of the body to store as ferritin, it accumulates in the liver as *hemosiderin*, a form of colloidal iron oxide in association with protein. The iron content of hemosiderin is nearly 35 per cent by weight.

Iron metabolism is disturbed mainly by the following causes :

1. Decreased formation of hemoglobin.
2. Decrease in circulating hemoglobin.
3. Abnormalities in the serum iron concentration.
4. Abnormal deposition of iron-containing pigment in the tissues.

The disorders of iron metabolism are : 1. Siderosis; 2. Nutritional siderosis; 3. Hemochromatosis.

1. Siderosis : If excessive amounts of iron get released in or introduced into the body beyond the capacity for its utilization, the excess is deposited in the various tissues, mainly in the liver. This may take place due to repeated blood transfusion, excessive breakdown of erythrocytes in hemolytic types of anemia and inadequate synthesis of hemoglobin as in pernicious anemia.

2. Nutritional siderosis : This disorder occurs among Bantus in South Africa. Bantus cook their food in large iron pots and consume iron-rich food. The absorption of iron appears to be high leading to the development of nutritional siderosis called Bantu siderosis. Livers of the Bantus have large amounts of iron.

3. Hemochromatosis : Hemochromatosis is a rare disease in which large amounts of iron are deposited in the tissues, especially the liver, pancreas, spleen and skin producing various disorders. Accumulation of iron in the liver, pancreas and skin gives rise to hepatic cirrhosis, bronze diabetes and bronze-state pigmentation respectively.

Iron Deficiency Anemia

Iron deficiency anemia is widely prevalent among children, adolescent girls and nursing mothers. The hemoglobin content of the blood is 5 to 9 g/100 ml.

Women of Child Bearing Age : The clinical symptoms are breathlesseness on exertion, giddiness and pallor of the skin. In severe cases, there may occur edema of the ankles.

Weaned Infants and Young Children : The hemoglobin level is 5 to 9g/100 ml blood. The children are dull, and inactive and show pallor of the skin. The appetitie is poor and growth and development get retarded.

28.10 Copper

Physiological Functions

Copper is necessary for the activity of cytochromes, catalase, tyrosinase, monoamine oxidase and ascorbic acid oxidase. Traces of copper are needed for normal synthesis of hemoglobin. The erythrocytes are having a colourless copper containing protein called erythrocuprein in small amounts. Cerebrocuprein is a copper containing protein present in the brain. Erythrocuprein, cerebrocuprein and another copper protein hepatocuprein in liver are all now identified to be an enzyme-cytosolic superoxide dismutase. It is a protein having 32,000 molecular weight and contains two cupric ions and two zinc ions per molecule. Ceruloplasmin is a copper containing protein (bluish coloured) present in plasma. It is regarded to be identical with the enzyme polyphenol oxidase. It also helps in the conversion of ferrous iron of plasma to ferric iron and its incorporation into ferritin.

Serum is having copper bound to ceruloplasmin associated with the alfaglobulin fraction and also copper loosely bound to albumin fraction.

In the intestinal mucosal cell, copper is associated with a low molecular weight metal binding protein called "metallothionein." Ceruloplasmin is a copper containing glycoprotein which is synthesized in the liver. It is not a transport form of copper.

Sources

Richest Sources : Liver, kidney, other meats, shellfish, nuts, and dried legumes.

Poor Sources : Milk and its products.

Cow's milk	0.015 to 0.18 mg/L
Human milk	1.05 mg/L (at the beginning of lactation).
Human milk	0.15 mg/L (at the end of lactation).

Distribution : The adult human body is having 100–150 mg of copper.

Muscles	64 mg of the total
Bones	23 mg
Liver	18 mg

The concentration of copper in the fetal liver has been found to be 5–10 times higher than that in liver of an adult.

Daily Requirements

Adults	2.5 mg.
Infants and children	0.05 mg/kg body weight.

A nutritional deficiency of copper could not be demonstrated in man, although it has been suspected in case of nephorosis.

Blood Copper : The normal concentration of copper in serum is 90 μg/100 ml. Both the red blood cells and serum are having copper. 80 per cent of the red blood cell copper is present as superoxide dismutase (erythrocuprein). The copper in plasma exists in firmly bound and loosely bound forms. The firmly bound copper consists of ceruloplasmin. The loosely bound copper is called "direct reacting" copper and is loosely bound to serum albumin. The plasma copper levels increase in pregnancy due to their estrogen content. Oral contraceptives have a similar effect.

Absorption : Absorption of copper takes place in the human duodenum. 30 per cent of the normal daily diet of copper gets absorbed in the duodenum.

Excretion : Only 10 to 60 μg of copper gets excreted in normal urine in 24 hours. The daily biliary excretion of copper is 0.5 to 1.3 mg and 0.1 to 0.3 mg excreted across the intestinal mucosa into the bowel lumen.

Effects of Copper Deficiency

1. Although iron absorption does not get disturbed but the release of iron into the plasma is prevented because of the decreased synthesis of ceruloplasmin. As a result, hypoferremia occurs which gives rise to the depressed synthesis of heme developing anemia in severe deficiency of copper.

2. The experimental animals on a copper-deficient diet lose weight and ultimately die.

3. In copper-deficient lambs, low cytochrome oxidase activity causes neonatal ataxia.

4. Copper deficiency produces marked skeletal changes, osteoporosis and spontaneous fractures.

5. Elastin formation gets impaired in the deficiency of copper. As a copper containing enzyme plays an important role in the connective tissue metabolism, especially in the oxidation of lysine into aldehyde group which becomes necessary for cross-linkage of the polypeptide chains of elastin and collagen.

6. Copper deficiency causes myocardial fibrosis in cows. It is suggested that reduction in cytochrome oxidase activity may cause cardiac hypertrophy.

Disorders of Copper Metabolism

Wilson's disease (*Hepato-lenticular degeneration*) :

Wilson's disease is a rare hereditary disorder of copper metabolism. The following disorders are seen in this disease.

1. The absorption of copper from the intestine becomes very high (about 50 per cent); whereas 2 to 5 per cent copper gets absorbed in normal subjects.

2. Ceruloplasmin formation is very less. Hence, a greater part of serum copper remains loosely bound to serum protein — notably albumin and, therefore, copper can be transferred to the tissues, such as brain and liver or to the urine.

3. Excessive deposition of copper in the liver and the kidney causes hepatic cirrhosis and renal tubular damage respectively. The renal tubular damage causes the increased urinary excretion of amino acids, peptides and glucose.

Menke's Disease

Copper is taken up by the intestinal mucosal cells, but its passage into systemic circulation through the serosal aspect of the mucosal cell is blocked. Mental retardation, instability of body temperature, abnormal bone formation, susceptibility to infections and kinky, steely hair are some of the symptoms of this condition.

Copper Toxicity : Poisoning by copper salts causes diarrhoea with bluish green stools. Saliva also shows a bluish green colour. Acute hemolysis and renal impairment can take place.

28.11 Iodine

Physiological Functions

Iodine is needed for the formation of thyroxine and triiodothyronine hormones of the thyroid gland. These thyroid hormones are involved in cellular oxidation, growth, reproduction, and the activity of the central and autonomic nervous system. Triiodothyronine has been found to be more active than thyroxine in many respects.

Sources

Iodine is mainly needed for the synthesis of the thyroid hormones. A minimum of 25 microgram is needed daily. The National Research Council (U.S.A.) has recommended a liberal intake of 100-200 microgram. Sea water has a high iodine content. Hence, vegetables and fruits and other foods obtained near the seashore contain sufficient amounts of iodine to meet its requirements. But in places located too far inland or at very high altitudes, the iodine content of natural foods is low and has to be supplemented by adding small amounts of iodine to table salt.

Daily Requirements

Adults	100-200 μg.
In adolescence and in pregnancy	200 μg.

Distribution

The body normally is having about 10 to 20 mg of iodine 70 to 80 per cent of this in the thyroid gland. Muscles have large amount of iodine. The concentration of iodine in the salivary glands, ovaries, pituitary gland, hair and bile is greater than that in muscle.

All the iodine in saliva is inorganic, but most tissues have less amount of iodine in the inorganic form and most of the iodine is present in the organic form.

Absorption

Iodine and iodides get absorbed most readily from the small intestine. Organic iodine compounds (diiodotyrosine and thyroxine) are partly absorbed as such and a part is broken down in the stomach and intestines with the formation of iodides. Absorption also occurs from other mucous membranes and the skin.

Storage

90 per cent of the iodine of the thyroid gland is in organic combination and stored in the follicular colloid as "thyroglobulin" which is a glycoprotein of molecular weight 650,000 thyroxine, diiodotryosine and smaller amounts of triidothyronine.

On demand, these substances get mobilized and thyroxine as well as triiodo thyronine gets passed into the systematic circulation. They undergo metabolic degradation in the liver.

Excretion

1. Mostly inorganic iodine gets excreted by the kidneys, liver, skin, lungs and intestine and in milk.

2. About 10 per cent of circulating organic iodine gets excreted in feces. This is entirely unabsorbed food iodine.

3. Nearly 40 to 80 per cent is excreted in the urine; of which 20 to 70 μg daily in adults and 20 to 35 μg in children. The urinary elimination is largest when the intake is lowest.

4. Urine iodine gets increased by exercise and other metabolic factors.

Blood iodine : In the resting state, serum contains 4-10 microgram of iodide per 100 ml. Most of this is bound to protein (protein-bound iodine, PBI) and represents the iodine contained in the circulating thyroid hormone. The PBI levels increase in hyperthyroidism and decrease in hypothyroidism. Oral intake of iodides causes a rise of serum inorganic iodine. This is not protein-bound.

Metabolism : There is a total of 50 mg of iodine in the human body. Muscles contain about 50% of this. The thyroid contains as much as 20% of the body iodine. Skin and skeleton contain small amounts. The concentration in the thyroid is, however, the highest : 10-40 mg/100 gm. Thyroid iodine is mainly organic and is combined with the protein of the colloid as 'iodo-thyroglobulin.' Various stages of the hormone synthesis—diiodotyrosine, triiodothyronine and thyroxine—are all present in the protein.

The hormones are released as required, enter the systemic circulation, and are mostly degraded in the liver and excreted through the bile. Bile thus contains iodides and also some of the organic products of degradation of the thyroid hormones. In tissues, thyroxine is mainly converted to triiodothyronine and thyroacetate.

Iodine Deficiency in Human Beings

1. *In adults,* the thyroid gland becomes enlarge producing the disease *goitre.* If treatment is started very early, the thyroid becomes normal. If treatment is delayed, the enlargement of the gland persists.

2. *In children,* severe iodine deficiency causes the extreme retardation of growth which is known as *cretinism.*

Prevention of goitre : Goitre can be prevented by the regular use of iodide salt or iodide added to the drinking water in the concentration of 1 : 5000 to 1:200,000.

Goitrogenic Substances in Foods

Foods, such as cabbage, cauliflower and radish contain substances 1 to 5, vinyl-2-thio oxazolidone which react with the iodine present in the food and make it unavailable to the body. These substances are termed as "Goitrogenic" substances.

28.12 Manganese

Physiological Functions

1. Manganese has been reported to be essential for normal bone structure, reproduction, and the normal functioning of the central nervous system.

2. Manganese ions are known to activate glucosyltransferase which is concerned with the synthesis of the mucopolysaccharides of cartilage and also associated with the synthesis of glycoproteins (*e.g.,* prothrombin).

3. Pyruvate carboxylase and superoxide dismutase are having tightly bound manganese.

4. Arginase gets activated by manganese ions.

5. It activates isocitrate dehydrogenase and phosphotransferases.

6. Manganese ions act as cofactor along with glucose-6-phosphate dehydrogenase.

7. Manganese ions are known to inhibit lipid peroxidation reactions.

Sources

Rich sources : Nuts and whole grains.

Good sources : Vegetables and fruits.

Poor sources : Meats, poultry, seafoods and fish.

Distribution

The body of normal adult (70 kg weight) is having 12-20 mg manganese. It occurs in all tissues of the body. The kidney and the liver are the main storage organs for manganese. Mitochondria are the principal intracellular sites of manganese uptake.

Daily Requirement

In humans, deficiency of manganese is not known. The average dietary intake of 2.5-7.0 mg is generally found to be sufficient.

Manganese in Blood

Normal blood is having 4-10 μg/100 ml. In human serum, manganese has been found to a specific β-globulin.

Absorption

Manganese is readily absorbed in the small intestine. Only 3 to 4 per cent of manganese present in the diet gets absorbed.

Excretion

95 to 96 per cent dietary manganese is excreted in the feces. Only traces of manganese gets excreted in the urine.

Manganese Deficiency in Animals

1. In manganese deficiency, the animals give birth to young ones which develop ataxia. In more severe deficiency, sterility results. In poultry, egg production and hatchability gets decreased even in mild deficiency of the metal.

2. The livers of manganese-deficient rats contain large amount of fat. This fat accumulation is prevented by manganese or chlorine.

3. Liver arginase activity and blood phosphatase activity get reduced in manganese deficiency.

4. Bone deformities also take place in all animals in its deficiency.

Manganese Toxicity

Miners who inhale large amount of manganese suffer from chronic manganese toxicity. There occurs the development of hepatolenticular degeneration resembling Parkinson's disease.

28.13 Cobalt

Physiological Functions

It is a constituent of vitamin B_{12} which is necessary for the formation of normal blood cells. In some of the lower animals like the rat, cobalt itself stimulates erythropoiesis. It is also said to liberate the hormone 'erythropoietin' which stimulates erythropoiesis. In ruminants the micro-organisms inhabiting the gastrointestinal tract synthesize B_{12} from the rumen, but only if cobalt is present in the pastures.

Certain enzymes, such as methylmalonyl CoA mutase, methyltetrahydrofolate oxidoreductase, homocysteine methyltransferase, and ribonucleotide reductase need vitamin B_{12} for activity.

Sources

It is highly available in food.

Distribution

It occurs in all tissues in small amounts. The total body content of cobalt is about 1.1 mg. The highest concentration is found in liver, kidneys and bones. Most of the cobalt occurs in vitamin B_{12}.

Daily Requirement

Its requirement for man is very less. It is, if required, required as vitamin B_{12}. As little as 1 to 2 μg of B_{12}, containing 0.045 to 0.09 μg cobalt, has been found to be sufficient to maintain normal bone marrow function in pernicious anemia.

Absorption

Cobalt is readily absorbed from the small intestine (70 to 80 per cent). Only minute amounts occur in the tissues, cobalt administered orally as a soluble salt is poorly absorbed and therefore largely eliminated in the feces.

Excretion

About 65 per cent of the amount ingested gets excreted in the urine, the remainder in the feces. Injected isotopic cobalt gets eliminated rapidly and almost completely by the kidneys into the urine.

Cobalt in Ruminant Nutrition

Nutritional anemia in cattle and sheep living in cobalt poor soil areas can be treated successfully with cobalt. Microorganisms in the rumens of these animals employ cobalt to synthesize vitamin B_{12}.

Cobalt Toxicity

Cobalt administered in large amounts to man or animals becomes toxic. It develops a condition called polycythemia (increased number of erythrocytes in blood).

28.14 Zinc

Physiological Functions

It is an element essential for normal growth, reproduction and longevity of animals. It is a component of several enzymes — alcohol dehydrogenase, alkaline phosphatase, carbonic anyhydrase, procarboxypeptidase and retinal — retinene reductase. Zinc is necessary for maintaining the plasma concentration of vitamin A.

The retina contains a zinc metalloenzyme, *retinene reductase* which is required for the formation of retinene.

It is required for the preparation of insulin and increases the duration of insulin action when given by injection. Zinc is used in the β- cells of the pancreas to store and release insulin as required.

It is concerned with the healing of wounds.

It is essential for the normal growth and reproduction of animals.

Source

An average mixed diet contains 10-15 mg of zinc. This is more than adequate. Meat, liver, eggs, fish, milk and cereals are good sources. It is readily absorbed from the intestines. Excretion is mainly through feces. Even parenterally administered zinc is excreted through feces. Pancreas, liver, kidney and spleen show a high turnover rate of zinc. Pancreatic juice is rich in zinc.

Rich sources : Oysters and herrings.

Good sources : Meat, eggs, liver and milk.

Fair sources : Cereals, pulses, nuts, oilseeds, vegetables and fruits.

Distribution

It is widely distributed in the tissues of the body. The whole body (70 kg. weight) 1.4 to 2.3 mg zinc. 20 per cent of the total occurs in skin. A certain amount is also present in the bones

and teeth. High concentration of zinc are present in spermatozoa, prostate and epididymis. The highest concentration occurs in the choroid of the eye.

Blood Zinc

1. Zinc occurs in higher concentration in erythrocytes than in plasma.

2. Normal plasma has about 20 per cent of the zinc present in whole blood.

3. The concentration of zinc of human blood, plasma and erythrocytes are 0.8 mg, 0.12 mg and 1.44 mg/100 ml respectively.

4. About 3 per cent of zinc ion is present in leukocytes. In certain types of chronic leukemia, there occurs a marked fall in the zinc content of peripheral leukocytes.

5. Most of zinc in erythrocyte exists in carbonic anhydrase.

6. The plasma concentration of zinc of human falls to 10 per cent of the normal level during later part of pregnancy and among those taking oral contraceptives.

Daily Requirement

Breast-fed newborn Baby	0.7-5 mg
Infants	3-5 mg
Children	10 mg
Adolescents	13 mg
Adults	15 mg
Pregnancy	30 mg
Lactation	25 mg

Absorption

1. Zinc present in animal foods is well absorbed in the small intestine, especially from the duodenum.

2. Zinc present in cereals, pulses, nuts and oilseeds is poorly absorbed because of the presence of phytic acid which interferes in its absorption.

Excretion

1. Zinc given orally or by injection is mostly excreted in the feces.

2. Endogenous zinc gets secreted into the small intestine in the pancreatic juice.

3. 90 per cent of zinc intake by healthy adult human gets lost in the feces, about 5 per cent is excreted in the urine and 5 per cent retained in the body.

4. Excessive sweating in the hot climate brings about excessive loss of the metal.

Deficiency of Zinc

1. Zinc deficiency in man causes dwarfism and hypogonadism (retarded genital development).

2. There occurs loss of taste acuity.

3. There also occurs poor growth, loss of appetite and hypogeusia in young malnourished children with subnormal hair zinc levels.

4. The deficiency of zinc brings about hepatosphenomegaly, delayed closure of the epiphyses of the long bones, and anemia.

28.15 Fluorine

Physiological Functions

Traces of fluorine are absolutely necessary for the normal development of teeth and bones. It particularly has a protective action against the development of dental caries, particularly in infancy

and childhood. It is also necessary for the prevention of development of osteoporosis in adults, particularly in postmenopausal women.

It is, in combination with vitamin D, required for the treatment of osteoporosis. Sodium fluoride is a powerful inhibitor of the glycolytic enzyme enolase. Fluoroacetate acts as a powerful inhibitor of aconitase, of the citric acid cycle.

Fluoride ions are known to inhibit the metabolism of oral bacterial enzymes and diminish the local production of acids which are important in the production of dental caries. Fluorine forms a protective layer of acid-resistant fluoroapatite with hydroxyapatite crystals of the enamel.

Sources

For humans, drinking water forms the main source of fluoride.

Daily Requirement

Fluoride occurs in small amounts in normal bones and teeth. Drinking water having 1 to 2 ppm meets up the requirement of the body and prevents dental caries without producing any ill effect.

Distribution

It is found in many tissues, notably the bones, teeth and kidneys. The amounts of fluoride in the soft tissues are very low and do not increase with age. It remains mostly in the extracellular water.

Absorption

Soluble fluorides get rapidly absorbed from the small intestine.

Excretion

It gets excreted in the urine, in the sweat, and by the intestinal mucosa. Most of the fluoride that escapes retention by the bones and teeth gets excreted rapidly into the urine.

Abnormalities

1. Intake of excessive amounts of fluoride (3 to 5 parts per million) in childhood is responsible for *"dental fluorosis (mottled enamel)"*. The enamel of the teeth loses its lustre and becomes rough. Chalky white patches with yellow or brown staining appear on the surface of the teeth. The enamel becomes weak and in severe cases a profound loss of enamel with 'pitting' occurs which gives both surfaces a corroded appearance.

2. Highly excessive intake of fluorine (over 10 parts per million) causes increased density and hypercalcification of the bone of spine, pelvis and limbs. In addition, the ligaments of the spine get calcified and the collagen in the bone is also calcified. Neurological disturbances are common. Such individuals are crippled and cannot exhibit simple daily tasks, like bending, squatting etc., as the joints become stiff.

3. Drinking water having less than 0.5 ppm fluorine is responsible for dental caries in children.

Prevention of Fluorosis

Fluorosis can be prevented by removing fluorides from the water by treatment with activated carbon or by some other suitable absorbents.

28.16 Molybdenum

Physiological Functions

Molybdenum is an essential component of xanthine oxidase, aldehyde oxidase, and sulfite oxidase. It also occurs in nitrate reductase in plants, and nitrogenase, which functions in nitrogen

fixation by microorganisms. Traces of molybdenum are needed for the maintenance of normal levels of xanthine oxidase in animal tissues.

Sources

Good sources : Liver, kidney, pulses, cereals, and some green leafy vegetables.

Poor sources : Other vegetables and fruits.

Distribution

Small amounts of molybdenum occur in all tissues. Liver and kidney are having larger amounts than other tissues.

Daily Requirements

Adequate amounts of molybdenum occur in average diets. Therefore, exact requirement is not known.

Absorption and Excretion

About 50 to 70 per cent of the intake is readily absorbed in the small intestine. Half of the absorbed molybdenum gets excreted in urine.

Toxicity

1. Molybdenum-rich diet consumption brings about severe diarrhea and ill-health in cattle.
2. Rats, on high molybdenum diet, lose body weight with marked anorexia.

28.17 Selenium

Physiological Functions

Its requirement for the human is not yet proved. But it is essential for many animal species. A deficiency of selenium produces hepatic necrosis, muscular dystrophy, necrosis of the cardiac muscle, and several other disorders in various experimental animals.

Selenium is a component of the enzyme which converts the glutathione to its oxidized form. In the conversion, H_2O_2 is used up and its accumulation is prevented. It is also required in some of the immune mechanisms, the biosynthesis of ubiquinone and the biosynthesis of ATP in mitochondria, The renal, cortex pancreas, pituitary and liver contain high amounts of selenium.

Selenium is essential for normal growth, fertility and for the prevention of a wide variety of diseases in animals, although not known as essential for humans.

It is involved in immune mechanisms, ubiquinone synthesis, and mitochondrial ATP biosynthesis.

Selenoprotein also functions in the reductive deamination of glycine.

Sources

Selenium is largely available in different foodstuffs. Its variation depends on the differences in soil selenium content.

Distribution

It is widely distributed in the animal body and highest concentration occurs in renal cortex, pancreas, pituitary and liver.

Daily Requirement

As average diet is having adequate amounts of selenium the requirement of it is not known.

Selenium Deficiency

1. Selenium deficiency causes necrosis of liver of rats.

2. Calves and lambs suffer from muscular dystrophy in selenium deficiency.

3. Chicks, on selenium-deficient diet, fail to grow and develop a diseased condition called exudative diathesis.

Relationship of Selenium to Vitamin E

Both selenium and vitamin E have been reported to be essential for curing certain diseases in experimental animals. When animals are given adequate amounts of vitamin E, selenium deficiency causes the following signs and symptoms.

1. Retardation of growth and muscular wasting in *rats.*

2. Retardation of growth and fertility in *chicks.*

These symptoms can be cured by the administration of both selenium and vitamin E because of their close metabolic relationship.

Toxicity

1. Chronic selenium poisoning causes development of "alkali disease." The symptoms of alkali disease include dullness, lack of vitality, roughness of coat, loss of hair from the body and tail, stiffness and lameness, cirrhosis of liver and anemia.

2. Acute selenium poisoning produces in animals salivation, grating of teeth, paralysis and blindness. Death occurs due to respiratory failure.

28.18 Chromium

Physiological Functions

It is able to potentiate the action of insulin in accelerating utilization of glucose in animals and humans. It has been found to be effective in improving glucose tolerance in some patients suffering from diabetes mellitus. It also maintains the normal cholesterol level in blood of rats. It regulates the incorporation of certain amino acids in heart muscle in rats.

Sources

It is highly available in dietary foods.

Distribution

The chromium content of adult human body has been found to be 6 mg. It is widely distributed in tissues.

Chromium in Blood

Normal blood is having about 0.009 to 0.055 parts per million.

Requirements

As average diet meets up the requirement, the exact necessity is not known.

Absorption and Excretion

It is readily absorbed in the small intestine. It gets mobilized from the tissues in response to glucose administration.

Chromium is mainly excreted in urine, a small amount gets lost in bile and feces.

Deficiency

Its deficiency has been characterized by impaired growth, disturbances in glucose, lipid and protein metabolism.

Toxicity

Excessive amounts of chromium cause growth retardation, liver and kidney damage in experimental animals.

29 Biochemistry of Blood

29.1 Introduction

Blood is a tissue that circulates in what is virtually a closed system of blood vessels. It consists of solid elements— the red and white blood cells and the platelets — suspended in a liquid medium, the plasma.

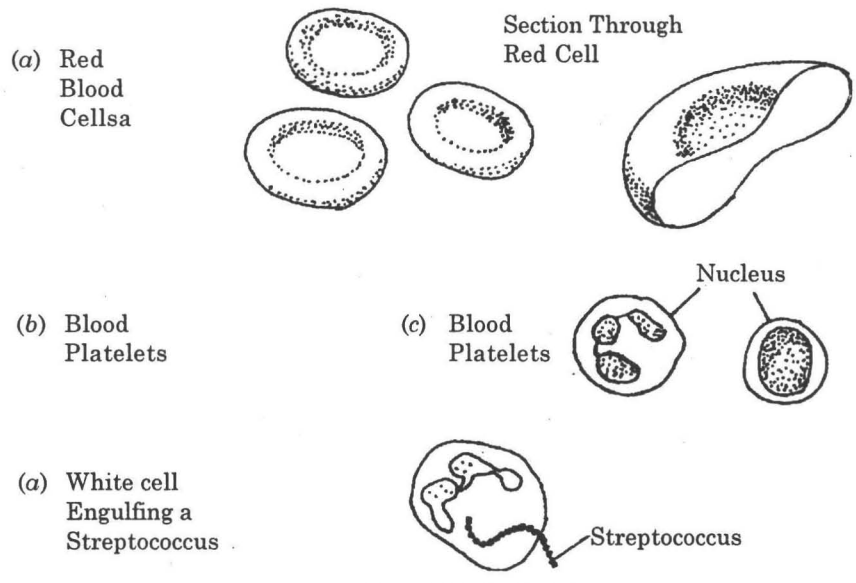


Fig. 29.1. Blood cells.

Once the blood has clotted (coagulated), the remaining liquid phase is called serum. Serum lacks the clotting factors (including fibrinogen) that are normally present in plasma but are consumed during the process of coagulation, serum does contain some degradation products of clotting factors — products that have been generated during the coagulation process and thus are not normally present in plasma.

The general features of blood are given as follows :

Specific gravity	1.060	
Blood Cells	40-45%	by volume
Platelets	200,000-4000,000	per c.mm.
Leukocytes (WBC)	5,000-10,000	per c.mm.
Erythrocytes (RBC)	4,500,000-6,000,000	per c.mm.
Plasma	50-60%	by volume
Solids	8-9	g/100 ml
Specific gravity	1.026	
Osmotic pressure at 37°C	7.6	atmospheres
pH	7.33-7.51	(mean 7.4)

29.2 Functions of Blood

(a) Homoeostatic Functions of Blood

Every living cell in the body is bathed with a *fluid*, the tissue fluid, which supplies it with the food and oxygen necessary for its metabolism and removes the waste products that could poison the cell if they are accumulated. Tissue fluid is derived from blood plasma; it contains a lower concentration of plasma proteins, but similar amounts of minerals, glucose and amino acids. Since the levels of these compounds in blood plasma are kept very precisely within certain limits by the liver and the kidneys, the body's cells are in contact with a liquid of almost constant composition. This provides them with the environment they need and enables them to live and grow in the most favourable conditions. By delivering oxygen and nutrients to the tissue fluid and removing the waste products of metabolism, the blood is important in maintaining the constancy of the internal environment.

In addition the blood has an important function in the maintenance of the body at an even temperature.

(b) The Blood as a Transport System

Blood circulates through every part of the body by the vessels of the *circulatory system;* on average, a particular red cell completes the circulation of the body in 45 seconds. This movement constantly brings fresh supplies of oxygen and nutrients to the tissue fluid as fast as they are used up, and removes the poisonous waste products before they can accumulate to harmful levels.

(i) Transport of oxygen from the lungs to the tissues : Oxygen is carried in the blood as oxyhaemoglobin, formed when blood is exposed to the relatively high concentration of oxygen in the lungs. Oxygen is appreciably soluble in water. It dissolves in the film of moisture lining the lungs, diffuses into the plasma in the capillaries there, and hence enters the red cells, where it reacts immediately with the haemoglobin in their cytoplasm to form oxyhaemoglobin. This prompt removal of oxygen from solution maintains a step diffusion gradient between the source of oxygen and the red cell, and thus ensures rapid diffusion of oxygen into the cell.

Oxyhaemoglobin is a bright red compound, while haemoglobin itself is dark red; oxygenated blood is thus a much brighter red in colour than deoxygenated blood. When blood containing

oxyhaemobglobin reaches a tissues where oxygen is being used up, the pigment breaks down into haemoglobin and free oxygen; the oxygen diffuses through the walls of the capillary blood vessels in the tissue, into the tissue fluid and so to the cells.

(ii) Transport of carbon dioxide from the tissues to the lungs : All living cells produce carbon dioxide as a waste product of their respiration. It diffuses from the cells into the tissue fluid and thence into the blood plasma. Some of it is carried in solution in the plasma as sodium hydrogen carbonate, and some in the cytoplasm of the red cells. It is released in the lungs where it diffuses through the lining of the lungs into the lung cavity, and is expelled from the body in the expired air.

(iii) Transport of nitrogenous waste from the liver to the kidneys : When the liver changes amino acids into glycogen in the course of deamination, the amino ($-NH_2$) part of the molecule is converted into urea, which is carried away from the liver in the blood. When the blood passes through the kidneys, most of the urea is removed; it leaves the body in solution in the urine.

(iv) Transport of digested food from the ileum to the tissues : The soluble products of digestion pass into the capillaries of the villi lining the ileum. They are carried in solution by the plasma and after passing through the liver enter the general circulation. Glucose and amino acids diffuse out of the capillaries and into the cells of the body. Glucose may be oxidized in a muscle, for example, and provide the energy for contraction; amino acids will be built up into new proteins and make new cells and fresh tissues.

(v) Distribution of hormones : Hormones are chemical compounds produced by certain glands, the endocrine glands. Each hormone affects the rate of one or more specific vital processes in the body they are carried away from the endocrine glands in solution in the blood plasma to every part of the body, although each hormone exerts its effect on only one organ or group of organs. For example, *adrenaline* secreted by the adrenal glands near the kidneys speeds up the heart-beat and the breathing rate, while *antidiuretic hormone* is secreted by the pituitary gland at the base of the brain, and controls the amount of water excreted by the kidneys.

(vi) Defence against infection : The blood of the higher animals contains large numbers of white blood cells of *leucocytes* which can engulf and destroy bacteria. In addition, the blood can produce chemicals called *antitoxins* which can effectively neutralize the poisonous proteins given out by the bacteria. Once an animal has recovered from an attack of a bacterial disease. Its blood is much better able to combat the particular bacteria producing that disease, and to neutralize their toxins : the animal is said to have acquired *immunity* to the disease.

29.3 Red Blood (*Erythrocytes*)

Red blood cells are minute discs of diameter ranging from 6–9 μ with an average of 7.5 μ, concave on both sides consisting of spongy cytoplasm contained in a thin elastic membrane. They have no nuclei. Their cytoplasm is coloured by a red pigment, *haemoglobin* which is a protein containing iron atoms in its molecule. It readily combines with oxygen in conditions of high oxygen concentration forming a compound called *oxyhaemoglobin.* Oxyhaemoglobin is unstable, and in conditions of low oxygen concentration it rapidly breaks down, reforming haemoglobin and releasing oxygen. These reactions are important in the transport of oxygen from the lungs to the tissues.

The circulating erythrocytes and the total mass of erythropoietic cells from which they are derived are termed as the 'Erythron.'

The cytoskeleton of the erythocyte gets formed by three groups of proteins called *spectrin, actin* and *ankrin.* Spectrin is similar to myosin of muscle. An interaction similar to actin and

myosin in muscle can take place between actin and spectrin in the erythrocytes. This interaction gets facilitated by ankrin. Because of this cytoskeleton, the erythrocytes not only maintain their normal biconcave disc shape but can also suitably alter their shape while passing through narrow capillaries to enable free passage.

Glycophorin is an important glycoprotein of the erythrocyte and occurs mainly in the cell wall. It is having sialic acid and other carbohydrate moieties. Differences in its structure are responsible for the three main blood groups — A, B and O which have the A, B and H or O antigens. The Lewis antigens Le^a and Le^b are not synthesized by the erythrocyte, but get added from the plasma onto the adult cells. They occur in the LDL and HDL (low and High density lipoprotein) fractions of plasma as glycosphingolipids.

I and P antigens are also glycoproteins. The M and N antigens occur in glycophorin itself. The M antigen having protein is termed as glycophorin A and the N antigen containing one is termed as glycoprotein B.

The erythrocyte cell membrane is readily permeable to (*i*) water, CO_2, urea, glucose (non-ionic molecules) (*ii*) HCO_3^-, Cl^- and OH^- (anions) and (*iii*) K^+ (cation). Only traces of sodium are present. (In plasma, mainly sodium is present and only small amounts of potassium). Similarly, magnesium is another cation which is present mainly in the cell while calcium is present mainly in the plasma.

The difference in the relative concentrations of Na^+ and K^+ between plasma and cell is maintained by mechanisms which are involving active transport mediated by enzymes called ATPases.

Two such enzymes have been identified — Na^+ — K^+ — dependent ATPase and Ca^{2+} — dependent ATPase. These function as Na^+ and Ca^{2+} pumps in removing these cations from the erythrocyte into the plasma.

The red cells are made in the red bone-marrow of the short bones such as the sternum (breast-bone), ribs and vertebrate. There are about 5 500 000 in a cubic millimeter of blood. A red cell lives for only about four months, after which it breaks down and disintegrates in the liver or spleen (an organ lying near the stomach). About 200 000 000 000 red cells are formed and destroyed every 24 hours, which means that about 1 per cent of all red cells are replaced daily; the number of red cells per cubic millimeter of blood remains, however, remarkably constant in a healthy person.

Mature mammalian erythrocytes contain no nucleus and no mitochondria. They metabolize only glucose, which they do only very slowly. One litre of packed human red cells consumes about 2 m moles glucose/hr at 37°C; red cells from several other mammalian species use glucose even more slowly than this. There is therefore a tendency to regard erythrocytes merely as inert bags, packaging haemoglobin molecules so tightly as to be almost at the limit of their solubility (the concentration within red cells is about 46 g/litre cell water, or 7 mM), as a means of avoiding the osmotic and viscosity problems that so much extra protein would produce, if it were simply dissolved in plasma.

The metabolism of red cells, however slow, does nevertheless have two important functions. The first is to keep its own membranes intact. There is a large number of inherent conditions in which the red cell membrane shows abnormal fragility, which may be manifested in abnormal shape (spherocytoma), shortened red cell life time (often accompanied by reticulocytsis), or in impaired response to a challenge by a drug or other chemical. Here we have space only to mention two such genetic defects.

1. Favism (Primaquine Sensitivity)

In this condition the glucose-6-phosphate dehydrogenase of red cells is much reduced in activity; typically it is about 15% of normal. In most cases the bearer of this defect is unaware of until he ingests one of a range of drugs, either anti-malarials such as primaquine, or anti-bacterial drugs such as sulphonamides. A component of the favabean (a common item of the diet in the Mediterranean) is another precipitating agent. The response is an acute haemolytic crisis. The biochemical reason is that the drugs cause oxidation of lipid components of the cell membrane and also oxidation of haemoglobin to methaemoglobin. The former is normally countered by glutathione peroxidase. NADPH is necessary to keep glutathione reduced, but the capacity of the red cells to do this is not great enough if G6PDH is defective.

2. Pyruvate Kinase Deficiency

Again there are many genetic variants of this defect. The red cell is peculiar in that its Embden-Meyerhof pathway of a bypass, which means that it does not necessarily produce more ATP than it consumes. In these circumstances partial loss of activity of one of the two enzymes producing ATP may mean that the Embden-Meyerhof pathway is no longer even self-sufficient in ATP, and it may not be able to maintain the flux of glucose to pyruvate which is necessary to provide reducing equivalents for glutathione and methaemoglobin reductases. Here again the deficiency, although ostensibly in energy metabolism, is fundamentally concerned with repairing damage to the membrane and to the cell contents resulting from the presence of O_2.

Methaemoglobinaemia

The second function of red cell metabolism is to prevent the accumulation of metHb. Not all methaemoglobinaemias involved red cell fragility; we have already seen that heterozygotes for HbM deficiencies have a normal life expectancy. Any reduction of the oxygen-carrying capacity of the blood must, however, compound cannot transfer either phosphate group to ADP, the result is loss of 1 ATP (per triose) in the energy balance of the complete pathway.

2, 3-Diphosphoglycerate is hydrolysed by a specific phosphates to 3-phosphoglycerate and inorganic phosphate, but as the activity of the phosphatase is low, 2, 3-DPG accumulates in the red cells. Its concentration in normal erythrocytes is about 5 mM, while that of ATP is 2 mM. Both these compounds are effector ligands for haemoglobin. Although the concentration of 2, 3-DPG does vary with O_2 demand, the changes much necessarily be slow, because of the very slow throughput of glucose in the system. For example, 2, 3 DPG doubles in response to living at lower O_2 tension (high altitude), but $t_{1/2}$ for the change is 15 hr. Changes in 2, 3-DPG in response to instantaneous changes in oxygen load are not to be expected.

29.4 White Blood Cells (Leukocytes)

There are various kinds of white blood cell. All consist of unpigmented cytoplasm, surrounded by a membrane, and containing a nucleus. There are between 4000 and 13000 in a cubic millimeter of blood, that is, there are about 600 red cells to every white cell. White cells are made in the bone-marrow, the lymph nodes and the spleen. Most of them are of a type called phagocytes which can move by a flowing action of their cytoplasm, rather like an Amoeba. They can ingest and destroy bacteria and dead cells by flowing round them and engulfing them in cytoplasm, and then digesting them by enzyme action, Phagocytes can pass out of blood capillaries by squeezing between the cells of the capillary wall; they move to the site of an injury or infection and accumulate there, destroying invading bacteria and damaged tissue, and preventing the spread of harmful bacteria to other parts of the body as well as accelerating the healing of the injured region.

The various kinds of leucocytes are as follows :

(i) Polymorphs : They possess a more active metabolism than erythrocytes. Glucose is completely metabolised to CO_2 and H_2O. They have glycogen and also several more enzymes than erythrocytes. Of particular interest is 'alkaline phosphatase' content. It is genetically controlled. The neutrophil alkaline phosphatase (NAP) content gets increased in Mongols and is virtually absent in chronic myeloid leukemia. Corticosteroid hormones increase NAP content. Polymorphs also contain small amounts of histaine. The cells have a short life. Usually they get destroyed by circulating toxins or by microorganisms in 6 to 8 hours. If not they become senescent in 24 to 30 hours and die.

(ii) Eosinophil leukocytes : They possess histamine and plasminogen. They survive only for a few hours in circulation. They get attracted to sites of histamine or 5, OH-tryptamine production which they somehow render physiologically inactive.

(iii) Basophil leukocytes : They have pepsin, 5, O-tryptamine and relatively large amounts of histamine. They are also termed as 'mast' cells.

(iv) Lymphocytes : Its two types can be distinguished on the basis of their life span. (1) Those which survive in circulation for only 2 or 3 days. (2) Those which survive for about 200 days. They are all indirectly produced by the thymus gland. In early fetal life, the lymphoid cells get 'seeded' from thymus in different parts of the body to become the lymphoid tissue of the adult. The thymus continues to exercise a humoral control over the lymphatic tissues even in the adult. The general metabolic pattern of the lymphocytes is similar to that of granular leukocytes. They are mainly concerned with the production of immunglobulins.

The lymphocytes responsible for formation of immunoglobulins (circulating antibodies) are termed as, "B-lymphocytes." There are others which are responsible for cellular immunity anti-tumour, anti-graft, anti-fungal etc. They are termed as the "T-lymphocytes."

(v) Monocytes : They are originated probably in the bone marrow and spleen. They mainly function as macrophages (engulf foreign materials) and can enter various tissues as and when needed to serve such functions. They take up antigents. Their association with the antigen, in some indirect and as yet ill-defined manner, facilitates the production of antibodies by the lymphocytes.

Monocyte (16-22 μ in diameter) is the largest cell in the blood. It has oral or horse-shoe shaped nucleus.

29.5 Blood Platelets (Thrombocytes)

These round or oval structures are very much smaller than red or white cells. There are about 400 000 of them in a cubic millimeter of blood; they are cell fragments without nuclei budded off from special, very large cells in the red bone-marrow, and they play an important part in the clotting action of the blood. The various characteristics of blood platelets are as follows :

1. They are unnucleated and so contain no DNA, but RNA is present.
2. They contain glycogen, ADP, ATP, and the enzymes of glycolysis.
3. Large amounts of catecholamines, 5 hydroxytryptamine (serotonin), and histamine are present in them.
4. Phospholipids are also present in the form of a lipoproteins which activate prothrombin in blood coagulation.
5. They also contain a contractile protein (thrombosthenin) which is involved in the process of clot retraction.

6. The life span of blood platelets is about 7-14 days.
7. They take part physically in hemostasis.
8. They are rich in serotonin and a protein which on release can cause an increase in vascular permeability.

29.6 Plasma

It is the liquid part of the blood. It is a solution in water of many compounds; it includes *mineral salts,* especially sodium chloride and sodium hydrogencarbonate, and *soluble proteins,* such as albumin, fibrinogen and the globulin antibodies. It also contains *hormones* and the products of the digestion of food, mainly *glucose* and *amino* acids, together with the waste products of metabolism, *carbon dioxide* and *urea.*

Blood serum is blood plasma from which the fibrinogen has been removed.

The main solids of plasma are the proteins which are almost 7-9 g per 100 ml. It is possible to separate out the individual proteins from mixture by different methods. 'Salting-out' methods use different concentrations of salt solutions (*e.g.,* ammonium sulfate, sodium sulfate and sodium sulfite) at which different proteins are precipitated and can be removed. More recently methods like electrophoresis and ultracentrifugation and ethanol fractionation (Cohn) have been utilised.

Plasma Proteins : When the early salting out techniques were used three proteins have been separated–albumin, globulins and fibrinogen. Globulins get precipitated by half saturation with ammonium sulfate whereas albumin gets precipitated only on full saturation. Fibrinogen gets precipitated by 1/5th saturation with ammonium sulfate. Among the globulins a fraction is present which can be precipitated on 1/3 saturation with ammonium sulfate. This is called the 'euglobulin' (true globulin) and the rest called 'pseudoglobulin' (false globulin). Euglobulin is insoluble in distilled water but soluble in dilute salt solutions, say NaCl; but pseudoglobulin is soluble even in distilled water.

Howe was able to fractionate serum proteins by employing different concentrations of sodium sulfate. Cohn used varying concentrations of ethanol at low temperature to separate out fractions of proteins which he called Fraction I, II and so on. Although each fraction is itself a mixture, yet it contains one of the proteins predominantly. Because the solvent can be readily removed by evaporation and the mild procedures used in the separation do not bring about denaturation, Cohn's method is useful for getting purified plasma proteins on a large-scale for therapeutic purposes.

Electrophoresis : This method was developed by Tiselius (1937) for analyzing plasma proteins. It is based on the principle that in solutions whose pH is above or below that of the isoelectric points or a mixture of proteins, the proteins will migrate in an electric field to the anode or cathode at different rates, the highest rate of migration is for that protein whose isoelectric pH is farthest removed from the pH of the solution and whose molecular size is smallest. It is possible to achieve this separation in a free solution kept in a U-tube by passing a current between two electrodes inserted in each limb of the U-tube. After running the current for several hours, the proteins which collect at different portions of the limbs of the U-tube are analyzed by suitable optical methods and photographing. A pattern of peaks is obtained as shown in Fig. 29.2, each peak representing one protein fraction. Further, it is possible to estimate the protein fractions quantitatively by measuring the areas under the curves for each peak.

It is possible to obtain similar results by using a filter paper strip to support the fluid instead of using an U-tube. The filter paper is held between two compartments having a buffer of suitable pH and the protein mixture (say plasma) is applied as a small dot or as a line across one end of

the filter paper. The current is passed through the filter paper by electrodes dipping into the two buffer chambers. The proteins migrate at different rates to the other end of the filter paper and in several hours time, they separate out sufficiently from one another to be identified by simple staining methods and may be estimated by employing suitable methods of densitometry of the stained paper or elution of each stained portion into suitable solvents and estimating colorimetrically. Instead of paper, several other supporting media such as cellulose acetate, starch etc., could be used for such electrophoresis.

The electrophoretic representation of normal human plasma is shown in Fig. 29.2.

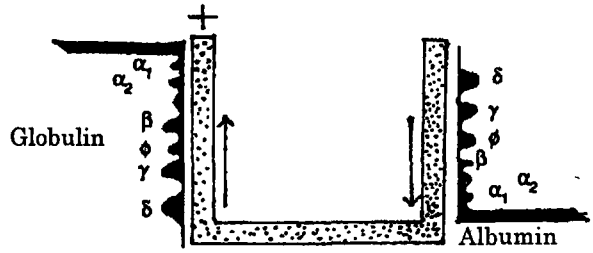


Fig. 29.2. Electrophoresis of normal human plasma.

By using such methods the serum could be separated into a number of fractions—albumin and three different types of globulines, α, β and γ. Depending on the sensitivity of the method used, the α globulin can be separated into an α_1 and α_2 fractions and β into β_1 and β_2 fractions. If plasma is used instead of serum, the fibrinogen fraction would be seen as a band between the β and γ globulin peaks.

When modern analytical techniques are used, more than eighty proteins can be identified in plasma. Many of them are found only in trace amounts and are either enzymes or transport proteins. Possibly several other proteins may exist in plasma which cannot be identified by the presently available methods.

The proteins which are readily identified by routine laboratory methods and which occur in more than trace amounts are listed in Table 29.1.

Table 29.1

Total proteins		6.3-7.8 g/100 ml
Albumins		3.2-5.1 g/100 ml
Alfa$_1$ globulins		0.03-0.39 g/100 ml
Alfa$_2$ globulins		0.28-0.74 g/100 ml
Beta globulins		0.69-1.25 g/100 ml
Immunoglobulins		
(gamma globulins)	I_gA	0.15-0.35 g/100 ml
	I_gG	0.80-1.80 g/100 ml
	I_gM	0.08-0.18 g/100 ml
Fibrinogen		0.20-0.40 g/100 ml

Albumin

About half of the total plasma proteins is albumin. It consists of 610 amino acids arranged in a single peptide chain. It has a molecular weight of about 69,000 and is synthesized in the liver. It is precipitated by full-saturation with ammonium sulphate. The normal concentration of serum albumin is 4.5 gm/100 ml. by precipitation method. It exerts 80 per cent of the colloid osmotic pressure of plasma. It plays an important role in the exchange of water between tissue fluid and blood. Its concentration decreases in severe protein deficiency, liver diseases and nephritis leading to the development of edema.

It has a low isoelectric pH (4.7). It migrates fastest in electrophoresis and precipitates last in salting out or alcohol precipitation methods.

It contains about 17 interchain disulfide bonds. It is simple protein (globulins are mostly glycoproteins). The molecule is ellipsoidal in shape (30 X 150A), and its solutions have low viscosity. It contributes 70-80% of the osmotic pressure of plasma proteins. It undergoes constant exchange with the albumin present in extracellular spaces of muscle, skin and intestines. It also helps in the transport of several substances like free fatty acids, bilirubin, Ca^{++}, and steroid hormones. Drugs such as sulfonamides, penicillin and aspirin also bind to albumin.

Alfa Globulins : They are glycoproteins. They are further classified into α_1, α_2 etc., depending on their electrophoretic mobility. α_1-Acid glycoprotein is having some structural resemblance to immunoglobulins. Alfa fetoglobulin occurs in high concentrations in fetal blood during mid-pregnancy. Normal adults have less than 1 µg/100 ml. It may increase during pregnancy. Some of the alfa globulins act as inhibitors of coagulation and also inhibit some enzymes like trypsin and chymotrypsin. There is a 'retinolbinding' protein and also a protein which, together with prealbumin, binds thyroxine. Ceruloplasmin is a copper containing $alfa_2$ globulin and is having eight sites for binding copper ions. It functions as a ferredoxidase and helps conversion of Fe^{++} to Fe^{+++}, which can be incorporated into 'transferrin.'

It also functions to transport Cu^{++}. In Wilson's disease, plasma ceruloplasmin level gets markedly decreased and copper levels of liver and brain get increased resulting in damage to those tissues. Haptoglobulins account for a fourth of the α_2 globulins. They can form complexes with hemoglobin. This is a mechanism which does not allow the urinary loss of hemoglobin released when erythrocytes are broken down.

Beta Globulins : Lipoproteins form an important constituent of this group. Transferin, the iron binding protein, is a beta globulin. Hemopexin is another which binds heme and prevents its excretion. C-reactive protein, it a betaglobulin. It occur in concentrations of less that 1.0 mg/100 ml in adult blood. It precipitates with a group C polysaccharide of Pneumococcus in the presence of Ca^{++}. Its levels in plasma get increased in acute infections.

β_2-microglobulin : It is having a low molecular weight and gets excreted in urine. It occurs in urine to the extent of only 0.01 mg/100 ml. It is also having close structural resemblance to immunoglobulins.

Immunoglobulins (Gamma Globulins)

The immune system consists of two entities :

1. The cellular immune system : This is mediated by the T cells (thymus derived lymphocytes) and is active against microorganisms, fungi, parasites, foreign tissues etc.

2. The humoral immune system : This is mediated by the B cells, (lymphocytes *not* derived from thymus), which secrete 'antibodies' or immunoglobulins when exposed to foreign substances called 'antigens'.

The immune system is spread diffusely throughout the body and is having 10^{12} cells in the spleen, liver, bone marrow, thymus, lymph nodes and in the circulating blood. The cells are having a mass of 2 kg and produce about 60 grams of protein for the immune system. It malnourished individuals, particularly in protein malnutrition, the immunity becomes low and the susceptibility to infection becomes high.

The different immunoglobulins can be broadly classified into three major classes—I_gG. I_gA and I_gM—and two minor classes—I_gD and I_gE. It is possible to distinguish them by their differences in sedimentation rates on ultracentrifugation, immunoelectrophoretic behaviours and

response to antigen stimulation. All of them are laving two identical light chains (L) and two identical heavy chains (H) held together as a tatramer (L_2H_2). In the light chain, the half towards the *c*-terminal is the *constant region* (CL) and the half towards the *n*-terminal is the *variable region* (VL). Further, about 3/4 of the heavy chain towards the *c*-terminals is the constant-region and 1/4 of the heavy chain towards the *n*-terminal is the variable region (CH and VH respectively). The constant region of the heavy chain can be further subdivided into three regions—C_H1, C_H2 and C_H3.

The terminal VL and VH regions (or domains) are the ones which determine the specificity of the antibody for the antigen and take part in binding the specific antigen. [See Figs. 29.3 (*a*) and (*b*)]. Digestion with papain will produce two antigen binding fragments (Fab) and one crystallizable fragment (FC). The cleavage occurs between C_H1 and C_H2 regions, which is called the *hinge region.*

The L chains are either kappa (K) or lambda (λ) and an immunoglobulin contains only one of them. The H chains can be any one of the five—gamma (γ), alfa (α), omega (ω), delta (δ) or eta (ε). The classification of the immunoglobulins is based on the H chains. Delta and eta chains have four CH domains instead of only three in the others. The main characteristics of the five classes of immunoglobulins are tabulated in Table 29.2.

Table 29.2

I_g type	*H chain*	*L chain*	*Carbohydrate content*
I_gG	gamma	kappa or lambda	4%
I_gA	alfa	kappa or lambda	10%
I_gM	omega	kappa or lambda	15%
I_gD	delta	kappa or lambda	18%
I_gE	eta	kappa or lambda	18%

It can be seen from above that all immunoglobulins are glycoproteins containing varying amounts of carbohydrates. The H chains have a molecular weight ranging from 50,000 to 75,000.

The L chains have a molecular weight about 23,000. While I_gG occurs are such, I_gA and I_gM may occur as polymers containing two or more identical subunits. The L and H chains are synthesized separately in the B cells and later assembled together. At least three genes take part in the synthesis of the L chain and four genes in the synthesis of the H chains. Man has the ability to synthesize antibodies to at least a million antigens.

Biological Role of Immunoglobulins

I_gM : It is the first antibody which gets formed in response to an antigen. It is having multiple, though weak, binding sites for antigens and can agglutinate cells with surface antigens. It is only slightly permeable into interstitial fluids and is impermeable to placental barrier. It therefore mainly occurs in the plasma.

I_gG : It is the next immunoglobulin which is formed in response to antigenic stimulation. Quantitatively, it is found in the largest amounts in plasma and its rate of synthesis is the highest (over 2 grams per day). It can enter the interstitial fluid and also cross the placental barrier and can provide immunity to the fetus. It readily binds to the antigen and also stimulates the complement system.

I_gA : It is produced still later. It is concentrated at sites of entry of antigens into the body—*e.g.,* the digestive and respiratory tracts. It is the main constituent of the colostrum milk (milk

secreted soon after child birth) and provides protection to the gastrointestinal tract of the new born infant against microbial infection through gastrointestinal tract.

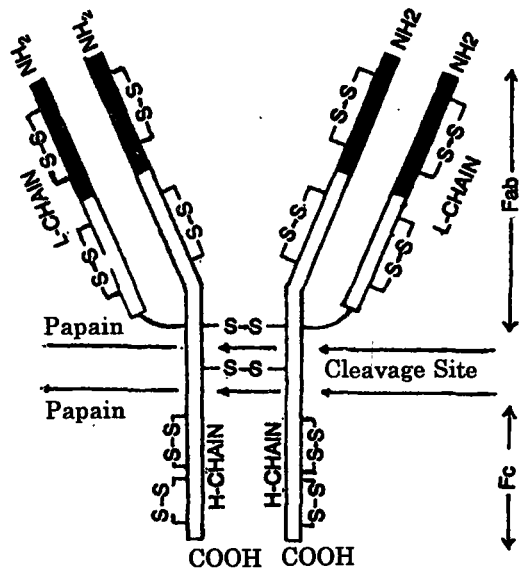


Fig. 29.3. (*a*) Structure of I_gG.

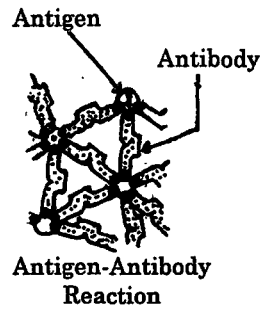


Fig. 29.3 (*b*).

Antibodies can be either *monoclonal* or *polyclonal.* Monoclonal antibodies have been reported to be highly specific and are able to bind a single antigenic determinant of an antigen. They get produced by cells derived from a single clone. Polyclonal antibodies are derived from multiple clones and are mixtures of several antibodies formed by different types of immunoglobulins.

Immunity

Immunity can be either active or passive.

Active immunity is acquired because of exposure to foreign cells or macromolecules either naturally or artificially (*e.g.,* smallpox vaccination, cholera inocculation). Immunity against smallpox, polio, typhoid and measles is an example of this type.

Passive immunity is acquired by injecting antibodies, prepared in another animal or human being exposed to the antigen earlier into the susceptible individual. Injections of diphtheria and tetanus antitoxins are examples of this type. Immunity offered by this method is transient and short lived compared to active immunity.

Complement System

This system consists of a group of proteins in the blood which complement the functions of the antibodies in eliminating antigens. They act by (*i*) enhancing capillary permeability and passage of phagocytic leukocytes to the site of antigen; (*ii*) stimulating phagocytosis and (*iii*) lysing microorganisms and tissue cells foreign to the organism. The components of the complement system are found in the plasma in an inactive (zymogen) form and have to be activated first. The proteins taking part in the pathway can be broadly classified into three groups :

1. The C_1 complex forms the recognition unit and binds to specific antigen-antibody complex on the cell surface.

2. The C_2, C_3 and C_4 form one unit called the activation unit. The binding of the C_1 unit to the antigen-antibody complex triggers on this unit to function.

3. C_5, C_6, C_7, C_8 and C_9 form the membrane attack unit. This gets activated by the activation unit formed in (2) above and attacks the cell membrane and is responsible for the death of the cell. Detailed chemistry and functions of these several components have been worked out.

Sometimes, even without the formation of the antigen-antibody complex and full activation, the complement system may directly recognize the invading microorganisms and act on them after the initial stages of complement activation only. This is termed as the *'alternative pathway.'*

Haptens *:* These are small molecules, which are poor antigens by themselves, when coupled with a carrier protein molecules, become potent antigens, haptens. Usually they are aromatic, charged molecules.

Fibrinogen : Its molecular weight is between 350,000 and 450,000. It is the precursor of fibrin (the substance of the blood clot). Like globulin, it is precipitated by half-saturation with ammonium sulphate. It normally constitutes 4-6% of the total proteins of the plasma. It is formed in the liver and its concentration in blood falls rapidly in the excessive destruction of liver tissue. It is a large asymmetrical molecule which is highly elongated having an axial ratio of about 20 : 1.

Site of Formation of Plasma Proteins

The plasmapheresis is a series of experiments wherein an animal is bled repeatedly, the plasma is separated from the cells by centrifugation, and the cells are reinjected suspended in saline. When these experiments are performed, the plasma protein level is very much reduced. It is possible in such an animal to find out the amount of each of the plasma proteins synthesized by finding out the amount of plasma that has to be removed to keep the level of that constituent constant in the animal. This amount of particular protein refers to the basal output by that animal.

It is reported that this basal output gets reduced to about one tenth the normal in an animal where the liver is short-circuited from circulation by anastomosing the portal vein to venacava (surgically known as ECK fistula). In cirrhosis of the liver (where the circulation through liver and the function of the liver are grossly deranged) the plasma protein levels are very much decreased. The proteins involved in all these conditions include the albumin, alfa and beta globulins and the fibrinogen. The gamma globulin actually increases. By these and other experiments, it is established that liver happens to be the sole source of fibrinogen and albumin. It is also the source for most of the α and β globulins. The gamma globulins are however derived mainly from the plasma cells and lymphoid tissues (reticuloendothelial system).

Alterations in plasma proteins are of diagnostic value. In most conditions involving changes in plasma proteins, there occurs a decreases in albumin and an increase in globulins (mainly gamma) leading to an alteration of the ratio albumin/globulin (normally 4.5/2.2 or 2 : 1 by salting out methods; and 3.8/2.9 or 1.3:1 by electrophoresis method). In severe cases, the ratio becomes reversed (A/G becomes 1/2 instead of 2/1). These alterations take place in diseases of kidney due to loss of protein (mainly the low molecular weight albumin) and in chronic infections due to increased amount of gamma globulin (antibody formation) and in diseases of the liver because of failure to synthesize albumin.

Functions of Plasma Proteins

1. Nutritive : Albumin is largely involved in the nutritive functions of the plasma proteins owing to its high concentration. It is effective as a source of protein in hypoproteinemic patients.

2. Water distribution : The colloid osmotic pressure of plasma proteins plays an important role in the distribution of water between the blood and the tissues. Plasma albumin is responsible

for this function due to its low molecular weight and quantitative dominance over other proteins. In kidney diseases where protein loss from the body is more, large amount of water moves to the tissues producing edema.

3. Buffering actions : The serum proteins can combine with acids or bases to maintain the pH of the blood. They act as acids and combine with cations (mainly sodium) at the normal pH of the blood.

4. Transport : The plasma proteins transport lipids and the fat-soluble vitamins (*e.g.*, A, D, & E). Bilirubin is associated with albumin and also with fractions of the α-globulins. Thus, bilirubin is transported along with them. β_1-metal combining globulins (Siderophilin) is responsible for the transport of iron in the plasma. Thyroxine is transported in association with an α-globulin (thyroxine-binding protein, TBP) and cortisol by a mucoprotein (transcortin). Many drugs and dyes are transported in the plasma in combination with albumin. Half of the calcium or plasma is bound to protein for transport. Hemoglobin liberated in intravascularly is carried to the reticuloendothelial system by complexing with the hepatoglobins.

5. Viscosity : Because of the presence of protein in plasma, it is a viscous fluid. The viscocity of the blood provides resistance to flow of blood in the blood vessels to maintain blood pressure at normal level.

6. Coagulation : Plasma contains prothrombin, fibrinogen and other factors involved in coagulation of blood.

7. Immunity : γ-globulins are present in plasma and these γ-globulins protect body against bacterial infections.

Enzymes : Plasma contains several enzymes of diagnostic importances in some diseases.

8. Fluid exchange : Plasma is having an osmotic pressure of about 6.5 atmospheres. A small portion of this (25 mm Hg, *i.e.*, about 1/30 atmospheric pressure) is contributed by plasma proteins. The other extracellular fluids are having much less protein content (about 10 mm Hg). The electrolyte content being the same, this difference in protein osmotic presssure makes the plasma to draw fluid from the extracellular space. This occurs at the venous end of the capillary. At the arterial end of the capillary, the hydrostatic pressure in the capillary which is about 30 mm Hg will be able to drive the fluid into the tissue spaces where the pressure is only 10 mm Hg. An effective exchange of fluid between tissue spaces and plasma is thereby maintained (see Figure 29.4).

In this function, albumin plays a greater role than globulins. As albumin is a smaller molecule, it exerts a higher osmotic pressure, weight per weight, compared to the heavier globulins. One gram of albumin per litre so serum exerts 5.54 mm Hg. pressure while one gram globulin exerts only 1.43 mm Hg.

Macroglobulinemia : I_gM (19S) fraction is much increased. Heavier macroglobulins (24-40S) may also occur. The condition takes place in neoplastic diseases, collagen disorders, chronic infections, amyloidosis and hepatic cirrhosis.

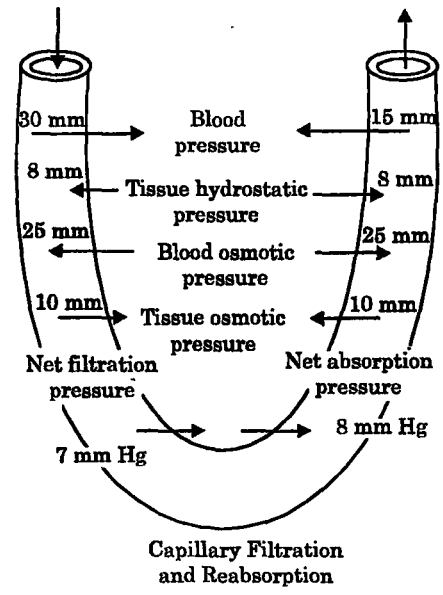


Fig. 29.4.

Multiple Myeloma : An abnormal protein, 'Bence Jones protein' occurs in blood and urine of people suffering from this condition. It is having a low molecular weight (45,000; 3.5S), and therefore readily escapes through the glomerular filter into urine. It is possible a dimer of the L-chains. It is easily identified in the urine by a simple test. On heating the urine to 50°-60° C, Bence Jones protein gets precipitated but when heated further, it dissolved again.

Cryoglobulins : These proteins are coagulated when the plasma or serum is cooled to very low temperatures. Traces are present even in normal individuals. They are increased in rheumatoid arthritis, lymphocytic leukemia, multiple myeloma and lymphosarcoma. The molecular weight varies from 165,000-600,000.

Enzymes such as amylase, lipase, transaminases and phosphatases occur in blood. They also show quantitative variations in disease and are of diagnostic importance. The clotting factors are regarded separately.

In addition to the chief proteins described above which are found in appreciable amounts, traces of several other proteins are also found which are detectable mainly by their action and isolated with great difficulty by use of highly sophisticated methods. This group will include dozens of enzymes and several factors which are concerned with coagulation or clotting of blood.

29.7 Blood Groups

Human blood can be classified into 4 main groups and several sub-groups. Agglutinogens are neutral nitrogenous mucopoly saccharides with molecular weights ranging from 200,000 to 300,000. The four main groups have been A, B, AB and O. The minor groups are M, N, P and Rh.

The plasma is having antibodies called agglutinin. The distribution of agglutinogens in RBC and agglutinins in plasma in the four groups are given below.

Blood group	*Agglutinogen in RBC.*	*Agglutinin in plasma.*	*Description of the group*
A	A	β(anti—B)	Aβ
B	B	α(anti—A)	Bα
AB	A and B	Nil	AB
O	Nil	α + β (anti-A + anti-B)	Oαβ

In order to determine the blood group of an individual, an isotonic saline suspension of RBC is mixed with a test serum having agglutinin α or agglutinin β on a slide. If no agglutination takes place, the cells separate and evenly distributed. If agglutination occurs, the cells clump together. The results can be interpreted as follows :

1. Blood of group A gets agglutinated by plasma of group B containing α-agglutinin.

2. Blood of group B gets agglutinated by plasma of group A containing β-agglutinin.

3. Blood of group AB gets agglutinated by the plasma of blood group A and B containing agglutinin β and α respectively.

4. Blood of group O is not agglutinated by the plasma of group A, B or AB. Therefore, persons of group O are termed as universal donor. Anybody can receive their blood.

Rh Blood Groups

It is an antigen of the Rhesus monkey and is found in the blood of 85% of white people and may be transmitted from father to child. The commonest Rh antigen is D and its antibody is

anti D. If the mother is Rh negative, she develops antibodies to it and these antibodies pass through the placenta to the fetus and bring about severe distraction of red blood cells in the new born child.

Homolysis or Laking of Blood

This term is used to describe the process by which hemoglobin from the stroma of the erythroyte gets released into the surrounding fluid (plasma or any other fluid in which the erythrocytes may be suspended). The cells maintain their shape and size when they are made to suspend a sodium chloride solution of 0.9% concentration. This is isoosmotic (isotonic) with the fluid inside the cell.

When kept in hypotonic solution water from the solution enters the cells due to the osmotic gradient and makes them to swell and with a sufficiently low concentration of sodium chloride, the swelling of the cell and stretching of the cell membrane will be such that hemoglobin escapes through spaces produced in the stretched membrane. The release of hemoglobin may also be because of the lowering of the concentrations of the other contents in the cell below a level optimal for binding the hemoglobin to the stroma. Normal erythrocytes commence to exhibit hemolysis in saline concentration of 0.45 to 0.39% and the process is complete (all hemoglobin comes out of the cell) at 0.33 to 0.3% saline concentration. A test performed to detect this range is termed as the 'fragility' test.

Besides salt concentration of the surrounding medium there are certain substances like chloroform and ether which dissolve away the lipids of the stroma and release the hemoglobin. Saponin, bile salts and detergents break down the lipid-protein complex of the stroma and brings about hemolysis. Toxic substances called hemolysins are found in snake venom and certain bacterial products which will also produce hemolysis of erythrocytes. Incompatibility of blood because of differences in blood groups will cause hemolysis in blood transfusions due to the presences of antigens.

It the cells are kept in a hypertonic solution, water passes out of the cell and the cells shrink and are irregular due to reduction in volume. The process is called crenation.

29.8 Hemoglobin

It is a conjugated protein which consists of 'HEME' as the prosthetic group. Heme containing proteins are characteristic of the aerobic organisms and are altogether absent in anerobic forms of life. Hemoglobin (R.B.C.), myoglobin (muscle), cytochromes, peroxidase and catalase (present in all cells) are all examples of heme containing proteins.

Heme

It is iron-porphyrin compound. The porphyrins are complex compounds having a tetrapyrrole structure, each pyrrole having the

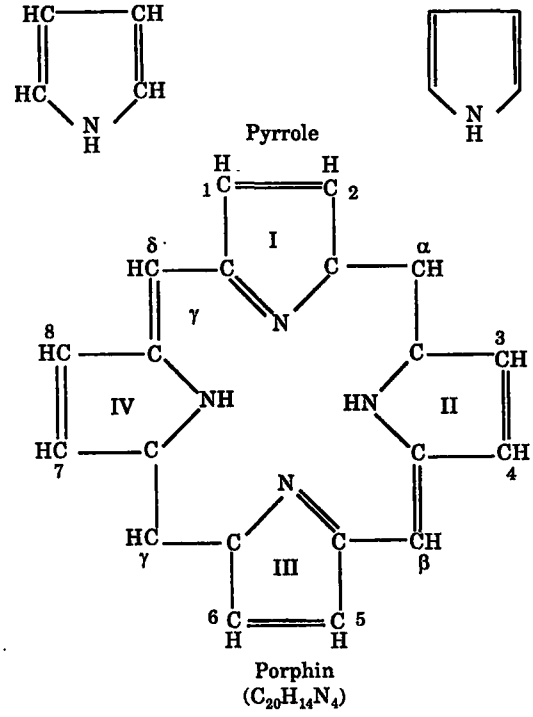


Fig. 29.5.

structure as shown in Fig. 29.5. Four such pyrroles get combined through —CH = (methylidyne) bridges to form a porphin.

The carbons in the four pyrrole rings (which are not linked with the methylidyne bridges) are numbered 1 to 8. The methylidyne bridges are termed as α, β, γ and δ.

The replacement of two hydrogen atoms in the —NH groups of pyrrole rings 2 and 4 may take place by ferrous iron (Fe^{++}) which will occupy the centre of the compound rings structure and establish linkages with all the four nitrogens of all of the pyrrole rings. If the iron is in the ferric state, it will carry a surplus + ve charge which gets balanced by taking a OH^- from the medium. It may also be balanced by other anions like Cl^- (the chloride compound is called 'hemim').

In addition to possessing ferrous iron in the centre, the hydrogens at positions 1 to 8 get substituted by different groups in different compounds. In the protoporphyrin which forms the parent compound of heme (protoporphyrin, Type III, series IX), the positions 1 to 8 have been substituted by methyl (—CH_3) vinyl (—$CH{=}CH_2$) methyl, vinyl, methyl, propionic (—CH_{2+}, CH_2, COOH), propionic and methyl groups in that order.

M	V	M	V	M	P	P	M
1	2	3	4	5	6	7	8

The porphyrin ring can be readily synthesized from simple substances (*e.g.*, glycine, succinate etc.) in the reticulo-endothelial cells.

Hemoglobin gets formed by conjugation of heme with basic protein called globin. Although the structure of heme is the same in hemoglobin from any animal source, the globin varies from species to species in its amino acid composition and structure.

Human hemoglobin is having 0.34% iron and is having minimum molecular weight of 16,400 which represents a single iron and hence a single heme unit per molecule. But determinations using ultracentrifugation and other techniques indicate a molecular weight of 65,000 which implies that four units (each containing one heme) form an aggregate molecule of hemoglobin. It is possible to break aggregate in concentrated urea solution and other salt solutions into the four units of globin peptide chains, each with one heme. Mild acid hydrolysis is able to remove the heme also leaving solitary globin polypeptide chains. The polypeptide can be broken into smaller peptides by enzymic hydrolysis and each such small peptide further analyzed to determine the amino acid sequence. By superimposing the information so gained, it is possible to determine the amino acid sequence of the entire globin polypeptide.

By such analysis, 4 different amino acid chains have been found to present in the globin moieties from different sources. They are named as the α, β, γ and δ chains. Human adult hemoglobin is having two alfa and two beta chains. The alfa chain is made up of 141 amino acids with valine at the *N*-terminal and arginine at the *C*-terminal.

The beta chain is having 146 amino acids with valine at the *N*-terminal and histidine at the *C*-terminal. Portions of the peptide chains have been helical while certain other portions have been not. Each of the four polypeptide chains is arrnaged in the forms of a tetrahedron with most of the polar and hydrophobic groups on the outer surface and the non-popular hydrophobic groups in the interior. This makes the protein readily soluble in water.

In addition each of the four polypeptide chains is able to form a pocket called the 'heme pocket'. The amino acids lining the pocket are strongly hydrophobic. A histidyl residue at position 87 of the alfa chain and position 92 of the beta chain get situated in their respective heme pockets. The fifth coordination valence of the heme iron is linked to the nitrogen of histidine in these positions.

Histidine is also present in the 58th position of the alfa chain and the 63rd position of the beta chain, quite close to the heme pockets of the respective chains. A sixth coordination valence of the heme iron, which, in reduced hemogloin gets loosely combined to a molecule of water, forms, in the presence of oxygen, a bond with the nitrogen of this extra histidine through oxygen.

$$HbH_2O + O_2 \longleftrightarrow HbO_2 + H_2O$$

Thus iron in heme is having six valencies as in ferrocyanide, $H_4Fe(CN)_6$.

Because of the hydrophobicity of the heme pocket water gets excluded and heme is thus kept in a medium of low dielectric constant. That is the reason why oxygen is able to complex with heme to form oxyhemoglobin without oxidizing heme iron to ferric. If the dielectric constant were to be high, heme iron would have been oxidized to ferric form and a highly toxic superoxide ion $(O_2)^-$ would have been formed.

The propionic acid side chains of heme in positions 6 and 7 of the tetrapyrrole structure are bound with lysine and arginine residues in the heme pocket. The alfa and the beta chains of the globin molecule are therefore eminently designed to provide attachment to four heme molecules and also to reversibly take up and part with oxygen by utilizing the sixth valence of heme iron. The globin molecule has also six —SH groups; two each in the alfa chain and one each in the beta chain.

A schematic representation of the hemoglobin molecule is depicted in Fig. 29.5. The overall size of the molecule is 64Å × 55Å × 50Å.

Variations in Hemoglobin Structure

In the normal adult, most of the hemoglobin (90-95%) is the adult type (HbA) having the structure described above with 2α and 2β chains in the globulin molecule. A small amount (2-3%) of a variant HbA, is also present and is made up of 2α and 2δ chains.

In the fetus, there is an altogether different hemoglobin called HbF (fetal hemoglobin) which is made up of two α and two γ chains. This is gradually replaced by HbA. (At birth HbA is 85% and HbF is 15%). The fetal hemoglobin is resistant to denaturation by alkali whereas adult hemoglobin is readily denatured. This property finds use in the identification of fetal hemoglobin.

Almost 300 variants of human hemoglobin have been found by electrophoretic and chromatographic methods.

Variations in the structure of hemoglobin can bring alterations in its solubility, affinity for oxygen and ease of denaturation in vivo. There are two pairs of genes for the synthesis of the alfa chain but only one pair for the synthesis of the beta chain.

Hemoglobins With Reduced Solubility

Sickle-cell hemoglobin (HbS) is the major abnormal hemoglobin in this group. The 6th amino acid of the beta chain is valine instead of the normal glutamic acid of HbA. Other hemoglobins like HbC, HbD, HbE and HbJ are also having slightly altered solubilities and bring about mild degrees of anemia.

The erythrocytes in sickle-cell anemia are sickle shaped instead of being biconcave discs. The sickling is better demonstrated when the HbS is in the reduced state. If the oxygen tension is low, HbS molecules aggregate to form filaments or tubular structures and precipitate from solution. Sickle cells are having shorter life span than normal cells and are more easily lysed. hence the anemia. The blood flow in the capillaries may be partially or completely blocked by the cells causing severe pains and damage to tissues. Erythrocytes of people with sickle cell trait are having both HbA and HbS. In those suffering from sickle cell disease, only HbS is present. The eryhrocytes in sickle cell trait seem to be more resistant to malaria than normal.

Hemoglobins with Altered Oxygen Affinity : Over 20 hemoglobins have been described, mostly named after the place they were first reported from Hb Chesapeake, Hb Kansas etc. The structural changes may involve the contacts between the alfa and beta chains, the DPG binding site or the heme pockets.

Some of the hemoglobins readily get oxidized (Fe^{++} converted to Fe^{+++}) to form methemoglobin (HbM). The condition is termed as methemoglobinemia. Five different types of HbM are known to exist, differing from normal hemoglobin in their ready convertibility to methemoglobin. This is because of replacement of an amino acid (histidine or valine) in the heme pocket by tyrosine or glutamic acid.

Unstable Hemoglobins : Some hemoglobins undergo in vivo oxidation and are precipitated to form 'inclusion bodies' or 'Heinz bodies'. Hb Hammersmith refers to one such example.

Thalassemia

The condition is no named, because it is generally caused in the Mediterranean countries (Thalassa means 'sea').

Normally the rates of synthesis of alfa and beta chains of hemoglobin have been equal. In thalassemia, the rate of synthesis of one of the chains is low with the result, the other chain which is present in excess is precipitated. In α-thalassemia, alfa chains are not synthesized adequately and hence beta chains get precipitated. In β-thalassemia, it is the reverse. The life span of the erythrocyte is much reduced and severe anemia gets developed. If alfa chains are not syntheiszed at all, a condition called *'hydrops fetalis'* gets manifested. The fetus dies in utero or soon after birth.

Hemoglobin A_{1c} (Glycosylated Form of Hemoglobin A_1)

In this, *n*-terminal valine of the beta chain of HbA_1 undergoes combination with 1-amino, 1-deoxy fructose. In normal persons, HbA_{1c} forms 3-5% of total hemoglobin. But, in diabetic subjects, it may form 6-15%. The level of glycosylated hemoglobin is an index of the level of blood glucose in the preceding several weeks and hence refers to better measure of the diabetic state than a single blood sugar estimation.

Properties of Hemoglobin

Acids hydrolyze and separate globin from heme. The heme gets further oxidized to ferriheme. In the presence of HCl this forms ferrihemechloride (hemin) which forms characteristic brown rhombic crystals which are readily identifiable under the microscope.

$$Hb + HCl \longrightarrow \text{Ferroheme} + \text{Globin}.$$

$$2\ \text{Ferroheme} + HCl + 1/2O_2 \longrightarrow \text{Ferriheme chloride (hemin)} + H_2O.$$

If an alkali is used for hydrolysis, it yields ferrihemehydroxide or alkali hematin.

If ferriheme undergoes combination with globin, the compound formed is methemoglobin. Some drugs like nitries, chlorates and ferricyanide oxidize hemoglobin in vivo and convert it to methemoglobin. The methmoglobin cannot function in the transport of oxygen.

Oxyhemoglobin

The most vital property of hemoglobin is its ability to readily form a complex with oxygen when exposed to high oxygen tensions and to dissociate equally readily and part with the oxygen when it is exposed to low oxygen tensions in the medium. The oxygen is only held loosely to the heme molecule by a redistribution of charges in the molecule. It is therefore termed as oxygenation as distinct from oxidation.

$$Hb + O_2 \longrightarrow Hb\ O_2$$

At an oxygen tension (partial pressure) of 100 mm Hg, the hemoglobin gets 95-96% saturated with oxygen. This is what happens to it in the lungs where alveolar pO_2 is about 100 mm Hg. In the arterial blood, the pO_2 is over 90mm Hg and at this pO_2, the hemoglobin is still over 90% saturated and carries 19.6 ml of O_2 per 100 ml blood. In the tissues the O_2 tension in it falls to about 10% and the passage of blood through them makes the O_2 tension in it to fall to about 40% (venous blood). At this pressure Hb is only 75% saturated and is able to retain only 12.6 ml oxygen and parts with the remaining 7 ml to the tissues.

The plotting of per cent saturation of Hb under various partial pressures of O_2 are known as oxygen dissociation curves (see Fig. 29.6). The dissociation also depends on the pCO_2 tension. Under physiological conditions of 37°C temperature and pCO_2 of 40 mm Hg the dissociation curve is S-shaped. It is reslatively flat between pO_2 100 mm to PO_2 60 mm and shows over 90% O_2 saturation, thereby ensuring adequate oxygenation of blood even under suboptimal, anoxic, conditions.

In the curve there is a sharper bend between pO_2 60mm to 40 mm during which it falls from 90% to 75% O_2 saturation, thereby facilitating rapid dissociation of HbO_2. An increase in CO_2 over 40 mm will shift the curve to the right and vice versa.

The Hb can also combine, with other gases, and in some cases with greater avidity. Carbon monoxide is having about 200 times as much affinity for Hb as O_2 and forms carboxyhemoglobin. $Hb + CO \longrightarrow HbCO$.

Hence CO is highly poisonous if breathed. Prolongled breathing of pure O_2 is needed to displace the CO from Hb.

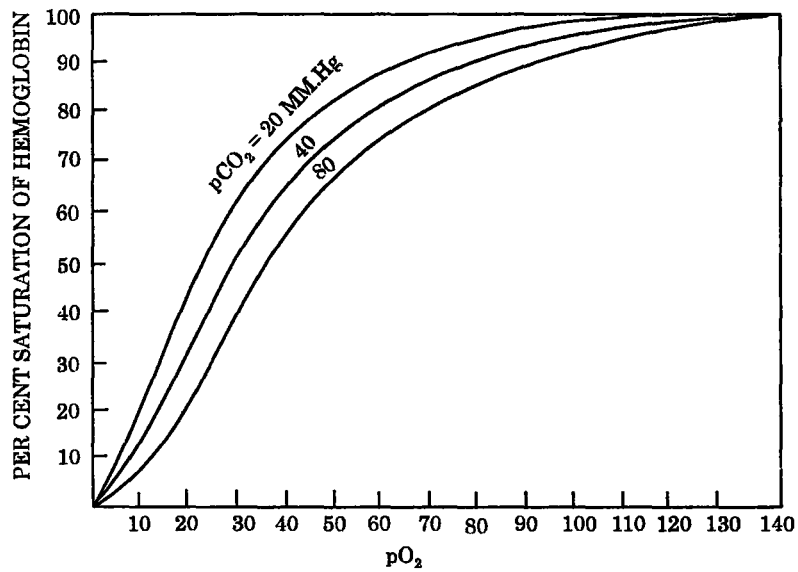


Fig. 29.6. Oxygen dissociation curves.

Carbamino Hb : The hemoglobin can directly combine with CO_2 to form carbamino hemoglobin.

Buffering : As it is a protein, it can also act as a buffer. This is true for reduced as well as oxyhemoglobin, both of which can be regarded as weak acids, HHb and $HHbO_2$.

They can therefore from buffer systems thus.

$$\frac{Hb}{HHb} \text{ and } \frac{HbO_2^-}{HHbO_2}$$

Also $HHbO_2$ is a stronger acid (pK = 6.60) than HHb (pK = 7.93). The conversion of the stronger acid $HHbO_2$ to the weaker HHb in the passage through the capillaries of the tissues

will itself make the weaker HHb to take up about 0.7 m eq. of H^+ from the tissues where it gets liberated from $H_2CO_3 \longrightarrow H^+ + HCO_3^-$.

This is termed as isohydric trnasport of CO_2 (without change in pH). The H^+ is taken up by the reduced Hb and the HCO_3^- passes into plasma in exchange for chloride which enters the erythrocyte (on account of Donnam membrane effect).

By the two mechanisms (isohydric transport and carbamimo hemoglobin formation) hemoglobin helps in the transport of a major portion of CO_2 formed in the tissue.

Sulfhemoglobin : When oxyhemoglobin is exposed to H_2S, sulfhemoglobin is formed.

$$HbO_2 + H_2S \longrightarrow HbS + O_2$$

As this reaction is not reversible, Hb S is no longer useful for transport of O_2.

29.9 Coagulation of Blood

If blood is drawn and kept in a container outside the body, it forms a solid mass in a very short time. This is called clotting of blood. In the course of a few hours a clear straw coloured liquid exudes from the red mass. This is termed as serum as distinguished from plasma which is obtained by centrifuging a sample of blood treated with an anticoagulant and collecting the clear supernatant fluid.

Howell originally described clotting to be involving 3 phases :

Stage I : Thromboplastin gets liberated from injured tissue or shed blood.

Stage II : Now the thromboplastin in the presence of ionic calcium acts on prothrombin a protein present in blood plasma and is able to convert it to thrombin.

Stage III : The thrombin in turn acts on fibrinogen, the soluble protein present in plasma and converts it to fibrin which is insoluble and precipitates out as a network of elongated thread such as fibres enmeshing the cellular and liquid components in the mesh work and yielding it a solid appearance.

Subsequently the fibres shrink and the mesh gets closely knit, still holding the cellular elements, but exuding out the liquid with its dissolved proteins and other organic and inorganic constituents. This fluid is termed as the serum.

This simple description is involving only 4 principal factors— thromboplastin, calcium, prothrombin and fibrinogen. However, it is now complicated by the discovery of several new factors and by an increasing insight into the detailed mechanisms involved in the clotting process.

Arrest of bleeding is termed as *hemostatis.* This involves the following long phases.

1. First of all vasoconstriction of the injured vessels takes place thereby reducing the blood supply to the area.

2. Then there occurs the formation of a loose plug of platelets—a *white thrombus*—at the site of injury. Collagen fibres exposed at the site of injury act as scaffolding on which the platelets get adherent and get disrupted releasing serotonin, epinephrine, prostaglandins and metabolities like thromboxane and ADP. These products increase the adhesiveness of the platelets and more platelets get deposited to form the platelet plug. Determination of bleeding time measures these two phases.

3. Now there occurs the formation of the '*red thrombus*' (blood clot).

4. Finally, there occurs partial or complete dissolution of the clot.

If a clot gets formed in an intact blood vessel without an apparent injury, it is termed as a *thrombus.* Many factors have been described (thirteen to date) which have a role in the clotting

process. They are designated by a common name as well as by a number. Many of them exist in circulation in an inactive form and have to be activated so that they could participate in the clotting process. Activation usually involves proteolytic removal of a segment or segments from the molecule. In fact, many of the factors are themselves *serine proteases* and help in activating each other, in a cascade like fashion.

The blood clotting factors are mentioned below :

Factor	*Name*
I	Fibrinogen.
II	Prothrombin.
IV	Calcium.
V	Labile factor, proaccelerin, accelerator (AC) — globulin.
VII	Proconvertin, serum prothrombin conversion accelerator (SPCA), cothromboplastin, autoprothrombin I.
VIII	Antihemophilic factor, antihemophilic globulin (AHG).
IX	Plasma thromboplastin component (PTC) (christmas factor).
X	Stuart-Power factor.
XI	Plasma thromboplastin antecedent (PTA).
XII	Hageman factor.
XIII	Laki-Lorand factor (LLF).

These factors are now described as follows :

Factor I, Fibrinogen : It is a soluble glycoprotein having molecular weight 340,000. It is made up of 6 polypeptide chains—two alfa, two beta and two gamma chains. The alfa, beta and gamma chains get linked lengthwise by -S-S-linkages. Two such chains are linked again by -S-S bridges. At the junction point, a swelling called the *disulfide knot* exists. The terminal portions are helical structures and also form two swellings, one at each end. The head ends carry a high negative charge because of presence of aspartate, glutamate and tyrosine residues in those portions. This does not allow these fibres from combing together to form a polymeric structure (the clot). They also keep the fibrinogen molecules in solution. Fibrinogen is synthesized in the liver.

Factor II, Prothrombin : It is also synthesized in the liver. Its molecular weight is 72,000. It is a single chain glycoprotein. At one end, it is having upto 14 molecules of gammacarboxyglutamic acid (GLa). Vitamin K is needed for their incorporation into the molecule and a deficiency of the vitamin therefore causes a deficiency of prothrombin. Factor Xa (active Stuart Factor) is able to convert prothrombin to thrombin by proteolysis at two points. Thrombin is the active form and can remove, by proteolysis, small peptides from the *n*-terminal ends of the alfa and beta chains of fibrinogen which contain the negative charges. As the remnant molecules, now called *fibrin* (monomeric form) are no longer soluble, they aggregate together and polymerize in the presence of calcium ions (factor IV), thereby forming the clot. (Fig. 29.7).

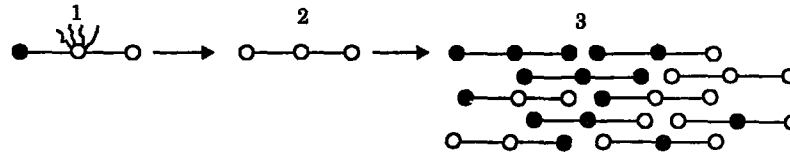


Fig. 29.7. Conversion of fibrinogen to fibrin.
1. Fibrinogen molecule
2. Fibrin monomer formed by removal of fibrinopeptide
3. Fibrin lattice formed by aggregation of the monomers.

Thrombin brings about proteolysis of fibrinogen as well as also converts Factor XIII (Laki-Lorand factor) to its active form-XIIIa. This is transglutaminase and brings about establishment of cross linkages between gamma-carboxyl groups of glutamine of one fibrin polymer with the eta-amino groups of lysine of a neighbouring fibrin polymer, thereby strengthening the clot and also causing *clot retraction.*

Factor IV, Calcium Ions : Ionic calcium is needed for several stages in the clotting process and is described at the appropriate places.

Factor V, Labile Factor, Proaccelerin, Accelerator (AC) Globulin : It is not having any enzyme properties. It gets activated to Va (formerly called Factor VI) by thrombin. In turn Factor Va becomes necessary for activating Factor X (Stuart factor) in the intrinsic system.

Factor VII, Proconvertin : *Serum prothrombin conversion accelerator (SPCA) :* It is an accessory protein which is activated to VIIIa by thrombin. In turn, it activates factor X to Xa in the extrinsic system.

Factor VIII, Antihemophilic Globulin (AHG); von Willebrand Factor : It is also an accessory protein which is activated to VIIIa by thrombin. It increases the rate of activation of factor IX (Christmas factor).

Factor IX, Christmas Factor : It is having glutamic acid carboxylate residues just like prothrombin and gets activated to IXa by factor XIa. It also converts the inactive factor X to the active form Xa. This is a very slow process, but gets enhanced 500 fold by the presence of factor VIII or VIIIa.

Factor X, Stuart Factor : It is the junction point for the extrinsic and intrinsic pathways. It is a serine protease and is having GLa residues. They help in clacium binding with acidic phospholipids of platelets. Factor

EXTRINSIC PATHWAY

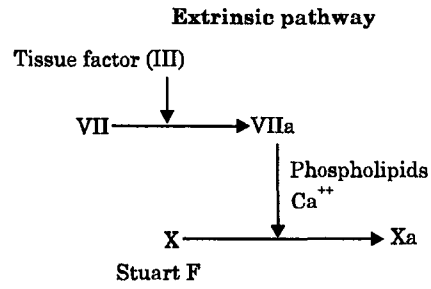


Fig. 28.8(*a*). Extrinsic pathway of blood coagulation.

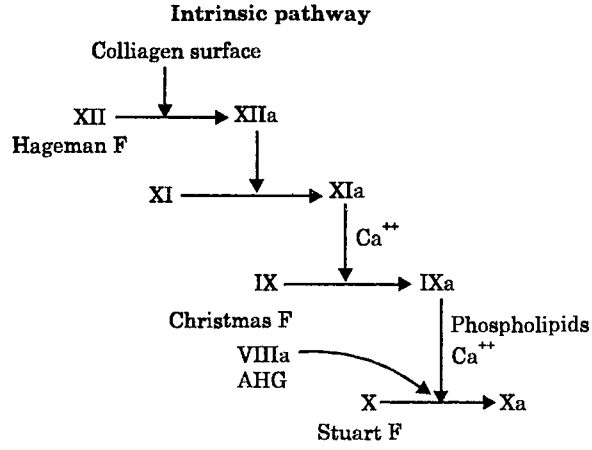


Fig. 29.8 (*b*) Intrinsic pathway of blood coagulation.

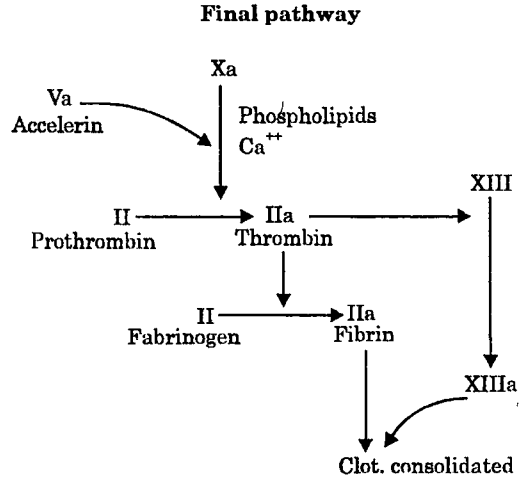


Fig. 29.8 (*c*) Final pathways of blood coagulation : Please note that the end product in both the intrinsic and extrinsic pathways in Factor Xa (active Saturat Factor). This forms the starting point for the final pathway.

X is converted to Xa in the extrinsic pathway by VIIa. Once some Xa gets formed, it autocatalytically converts large amounts of X to Xa.

Intrinsic Pathway : This is started with exposure of prekallikrein which is a high molecular weight kininogen factor XII and factor XI to an active surface—say collagen, in vivo and glass or kaolin in vitro. Kallikrein will convert XII to the active XIIa by proteolysis. XIIa releases bradykinin from high molecular weight kininogen and activates XI to XIa. All these, together with factors IXa, VIII or VIIIa convert factor X to Xa by proteolysis. Ca^{++} and phospholipids are also needed for the conversion.

Factor XI, Plasma Thromboplastin Antecedent, PTA : It is activated to XIa by factor VIIa. The XIa in turn activates factor IX to IXa.

Factor XII, Hageman Factor : It is activated to XIIa by contact with glass or by kallikrein system.

Factor XIII, Fibrin Stabilizing Factor : It refers to the zymogen form of a transglutaminase and gets activated to the zymase form, XIIIa, by thrombin. It is involved in the consolidation of the clot by cross linkages between the gammacarboxyl groups of glutamine and the eta-amino groups of lysine.

Factor XIV, Protein C : It is a zymogen of a protease, which on activation by thrombin to XIVa, inactivates factors V and VII by proteolysis.

Pre-Kallikrein : On conversion to Kallikrein by contact, it is able to activate factor XI and XII.

High Molecular Weight Kininogen : It is accessory protein factor for activation of factors XI and XII.

Plasminogen : It is the zymogen form of plasmin. It gets activated to plasmin by tissue factors and brings about fibrinolysis.

Factor III, Tissue Factor, Thromboplastin : Accessory protein from tissue, in the presence of phospholipids increases the extrinsic pathway of clotting.

What triggers clotting ?

Extrinsic Pathway : This gives rise to rapid coagulation. Thromboplastin a lipoprotein tissue factor, comes into contact with blood, on injury to endothelium or tissue. It activates Factor VII present in the blood to VIIa. The factor VIIa formed will in turn activate factor X to Xa.

Intrinsic Pathway : The initial reaction involves the exposure of factor XII to the negatively charged surface of collagen or activated platelets. Factor XII then gets hydrolyzed to XIIa which now activates factor IX to IXa in the presence of Ca^{++}. Factor IXa, in the presence of factor VIII or VIIIa, platelet phospholipids and Ca^{++} will activate factor X to Xa.

Final Pathway : In the intrinsic or extrinsic pathway, the activation of factor X to Xa is the end reaction. Thus formation of factor Xa refers to the junction point for the two pathways.

Factor Xa, in association with Va and Ca^{++} ions converts prothrombin to thrombin. Thrombin will now convert fibrinogen to fibrin monomers by proteolytic removal of fibrinopeptides from fibrinogen. Simultaneously, it activates factor VIII to VIIIa an active transglutaminase. The fibrin monomers get cross linked to form the fibrin polymer or the clot.

A summary of the intrinsic and extrinsic pathways of coagulation and the final pathway has been depicted in Fig. 29.8 (*a*), (*b*) and (*c*).

Inhibitors of Clotting

Several proteins are present in plasma which act as inhibitors of the proteases involved in blood clotting. *Antithrombin III* is the chief amongst them and inhibits all serine proteases including trypsin, chymotrypsin, plasmin, thrombin, factors IXa, Xa, XIa and XIIa.

Heparin is a very effective anticoagulant which acts mainly by activating antithrombin III.

Coumarin group of drugs (*e.g., Dicumarol*) act by interfering with vitamin K dependent carboxylation and conversion of glutamic acid to gamma-glutamyl carboxylate (GL to GLa). The production of prothrombin, Factors V, VII, IX and X get decreased. Thus all stages of coagulation get inhibited.

Orgal contraceptives may diminish Antithrombin III activity thereby favouring intramuscular thrombotic phenomena.

A deficiency of antithrombin III is also inherited as an autosomal dominant.

Protein C : Extension of the blood clot beyond the point where it is needed to cause hemostasis is prevented by trapping all the activated factors within the initial blood clot formed, so that they are no longer free to act on the circulating blood. In addition, there is a specific protein in the plasma-*protein C*- which gets activated by thrombin. The activated protein C destroys factors Va, VIIIa and Xa in vitro. It may exert the same action in vivo also.

Fibrinolysis

A proenzyme present in plasma—*plasminogen* gets activated to *plasmin* by kallikrein. The plasmin formed hydrolyzes fibrin to a number of small, soluble peptides. The clot is thereby dissolved. Protein C may also play a role in the conversion of plasminogen to plasmin.

Bradykinin refers to one of the byproducts of the action of killikrein on large molecules in the plasma called *kininogens* (M.W. 50,000 to 250,000 to 250,000). Bradykinin is a small peptide with only 9 amino acids. It dilates blood vessels, increases their permeability, constricts smooth muscle and is mainly responsible for bringing about intense peripheral and visceral pain stimulating the pain receptors.

Complement

The complement system also gets triggered into action on formation of factor XIIa.

Anticoagulants

In Vitro : Blood gets prevented from clotting in vitro mainly by preventing access to calcium ions. Removal of Ca^{++} by addition of oxalate, citrate, ion exchange resins and EDTA (ethylenediamine tetra-acetate) will either precipitate calcium as an insoluble salt or convert it into a non-ionizable salt and thereby prevent the coagulation process.

29.10 Lymph

The fluid in the lymphatic vessels is called *lymph.* Its composition is similar to that of blood plasma, but it contains less protein. No red blood cells are present, but it contains special white cells, *lymphocytes,* made in the lymph nodes and especially concerned with the production and transport of antibodies. After a meal containing fats lympth is a milky-white colour due to the fat droplets absorbed in the lacteals of intestinal lining which drain into the lymphatic system.

Lymph flows in one direction only, from the tissues to the heart. There is no specialized pumping organ; the flow is brought about partly by the pressure of the fluid accumulated in the tissues and partly by muscular exercise, the pressure of the contracting muscles around the lymphatics assisting the fluid through the vessels. Some of the lymphatics contain valves (Fig. 29.9), which prevent the lymph from flowing backwards, away from the heart.

Fig. 29.9. Deep lymphatic vessel cut open to show valves.

The intestinal fluid is collected by lymph capillaries which empty ultimately into the thoracic duct and right lymphatic duct which join the left and right subclavian veins and thus enters the systemic circulation. The daily lymph flow is 1 to 2 litres in the human adult. Diffusible non-electrolytes like urea and glucose are having the same concentration as in plasma. Diffusible non-electrolytes like K^+, Na^+, Cl^- and HCO_3^- tend to be somewhat higher than in plasma. This is because of the lower protein content of lymph which creates Donnan effect. The protein content of lymph varies with the location from which lymph is collected. It is highest in lymphatics of liver (about 6.0 gm %) and lowest in subcutaneous lymphatics (0.25 gm %). It averages about 3% in thoracic duct. The albumin/globulin ratio has been found to be much higher than in plasma.

30 Digestion, Absorption and Metabolism of Food

30.1 Introduction

If the food taken into the mouth is to be of any value to the body, it must enter the bloodstream and be distributed to the living cells. But a piece of meat or bread, for instance, cannot enter the blood unchanged : its insoluble constituents must be chemically altered to soluble compounds. *Digestion* is the breakdown of insoluble food materials, many of which have very large molecules, into soluble compounds having smaller molecules; these can pass in solution through the walls of the intestine and enter the bloodstream, by the process of *absorption*. Digestion and absorption both take place in the *alimentary canal* or gut (Fig. 30.1), a muscular tube running from the mouth to the anus. Some of its regions have specific functions and accordingly specially adapted structures. In its lining or *epithelium* are glands which produce some of the *digestive juices*, the fluids which bring about the breakdown of food; other juices are poured into the alimentary canal through ducts from glandular organs outside it. As the food passes through the alimentary canal it is broken down in stages until the digestible material is dissolved and absorbed. The undigested residue is expelled from the body through the anus.

30.2 Enzymes

Digestion of food is brought about by chemicals in the digestive juices called *enzymes*. Enzymes are catalysts, that is, substances which accelerate the rate of chemical reactions without altering the end products. All enzymes are proteins, and they occur in great numbers and variety in all protoplasm, controlling virtually all the chemical processes that take place in living things; without them these processes would be too slow to maintain life. The vast majority of enzymes are *intracellular*, that is, they carry out their functions in the protoplasm of the cell in which they

are made. Some enzymes, however, pass from the cells which have produced them to be used elsewhere, a process called *secretion.* These are called *extracellular* enzymes; bacteria and fungi secrete such extracellular enzymes into the media in which they are growing in order to accelerate the conversion of their food materials into soluble substances that can enter the cells. The higher organisms secrete extracellular enzymes into the alimentary canal for the digestion of food taken into it.

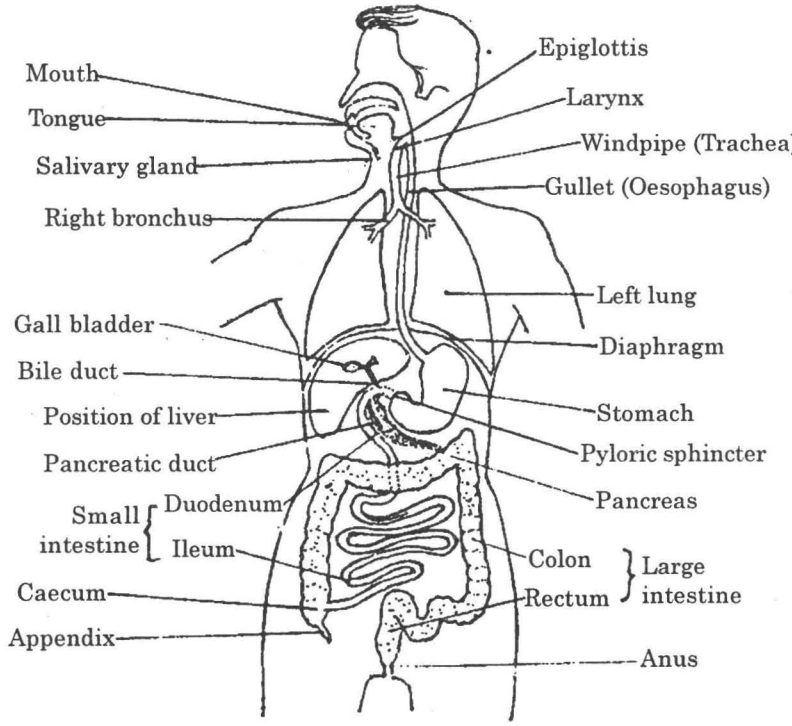


Fig. 30.1. Alimentary canal.

Every enzyme has the following characteristics :

(*a*) like all proteins it is destroyed by heat;

(*b*) it acts best within a narrow temperature range;

(*c*) it acts most rapidly at a particular degree of acidity or alkalinity (pH);

(*d*) it affects the rate of only one kind of reaction;

(*e*) since it affects only the rate of the reaction, it always forms the same end-product or products.

Digestive enzymes accelerate the rate at which insoluble compounds are converted into soluble ones. Enzymes which act on starch are called *amylases,* those acting on proteins are *proteinases,* and *lipases* act on fats.

30.3 Advantages of Digestion

1. The composition of the body proteins, fats and polysaccharides is not the same as that of proteins, fats and polysacchardies of food. Only the units of these complex substances are the same in the body and the blood. The food protein must be broken down into amino acids from which the tissue protein can be formed. Similarly, the polysaccharides and fats are formed in the body.

2. Disaccharides which are absorbed easily are used in the body as such and are excreted as foreign substances. But after digestion to hexoses, they are utilised in the body.

3. Digestion prevents the undesirable effects of the introduction of the foreign proteins into the blood converting them into amino acids which have no harmful effect.

4. It prevents the too rapid absorption of food and thus enables the blood to distribute the absorbed substances without undesirable effects.

5. The power of digestion increases the variation in our diet and our enjoyment.

30.4 Movement of Food into the Alimentary Canal

Ingestion is the act of taking food into the alimentary canal through the mouth.

Swallowing (Fig. 30.2) : In swallowing the following sequence of actions takes place :

(a) the tongue presses upwards and back against the roof of the mouth, forcing the pellet of food, called a *bolus,* to the back of the mouth or *pharynx;*

(b) the *soft palate* rises and closes the opening between the nasal cavity and the pharynx;

(c) the *laryngeal cartilage,* a ring of tough gristle-like material round the top of the trachea or windpipe, is pulled upwards by muscles so that the opening of the trachea lies beneath the back of the tongue : this opening is simultaneously constricted by the contraction of a circular *sphincter* muscle;

(d) the *epiglottis,* a flap of cartilage, directs food over the tracheal opening.

In this way food is able to pass over the entrance to the trachea without passing into it. The beginning of the action is voluntary, that is, it is under the conscious control of the will, but once the bolus of food reaches the pharynx swallowing becomes an automatic or reflex action.

Peristalsis is the way in which food is forced down the *oesophagus* or gullet into the stomach and subsequently through the lower regions of the alimentary canal. The walls of the whole alimentary canal contain muscle fibres which run both circularly and longitudinally. The circular muscles contract and relax in such a way that waves of contraction pass along the alimentary canal, pushing the food steadily onwards and preventing its movement in the reverse direction (Fig. 30.3).

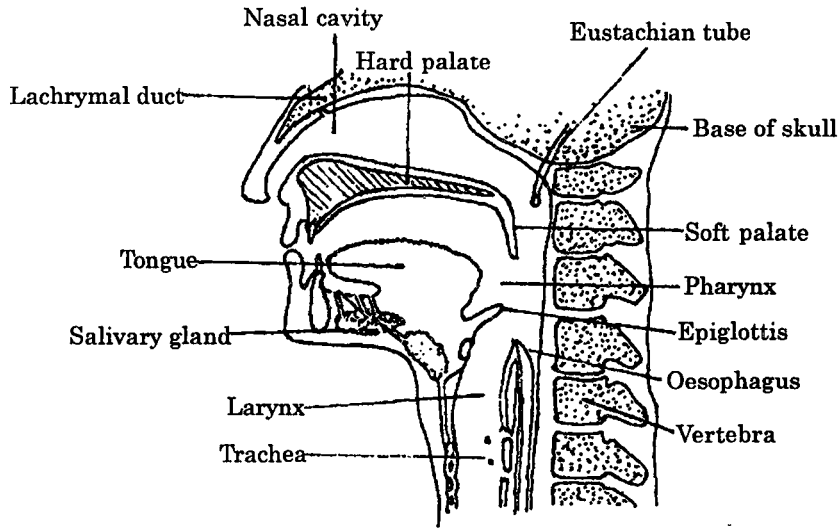


Fig. 30.2.

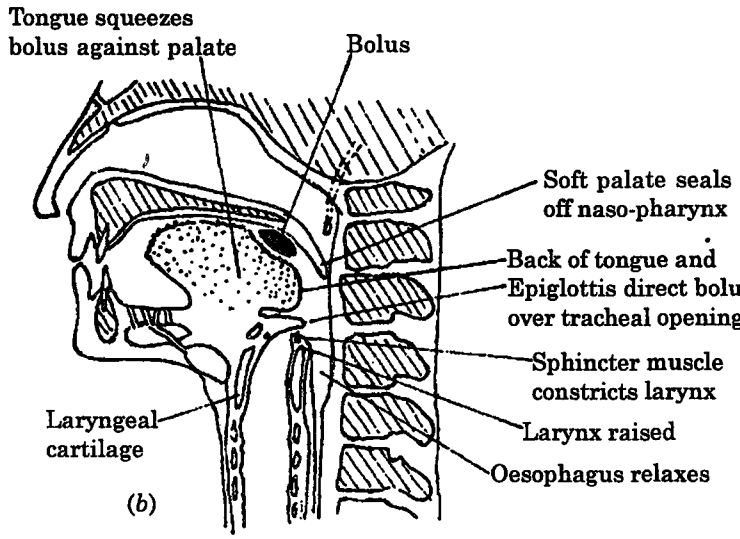


Fig. 30.2. Sections through head to show swallowing action.

Digestion in the Mouth

In the mouth the food is mixed with *saliva* and chewed or *masticated* by the action of the teeth and tongue; this softens it and reduces it to pieces of a suitable size for swallowing, and also increases the surface available for enzymes to act on. Saliva is a digestive juice secreted by three pairs of glands, namely, parotid, submaxillary and sublingual; the ducts of which lead into the mouth; an adult secretes 1 to 1.5 litres of saliva daily. The rate at which saliva is secreted increases when the body receives the stimuli of the taste, smell, sight or even the thought of food. It is a watery fluid, not particularly acid or alkaline, containing a little *mucus,* a somewhat viscous, slimy substance which helps to lubricate the food and make the particles adhere to each other. This process is called mastication. Saliva also contains an enzyme, *salivary amylase or ptyalin,* which acts on cooked starch (for example, in bread or boiled potatoes) and brings its breakdown to a soluble sugar called *maltose,* the molecule of which consists of a pair of linked glucose units.

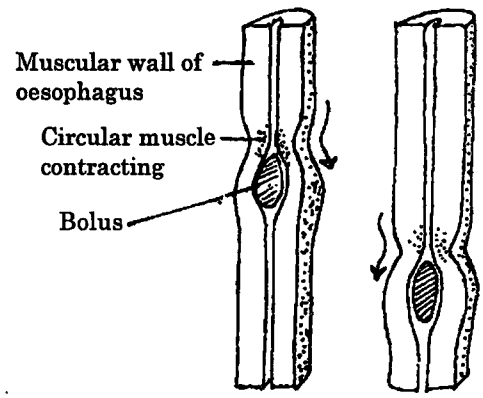


Fig. 30.3. Diagram to illustrate peristalsis.

The saliva is also a vehicle for the excretion of certain drugs (*e.g.,* ethanol and morphine), of inorganic ions such as K^+, Ca^{2+}, HCO_3^-, thiocyanate (SCN^-), and iodine, and of immunoglobulins (IgA).

The pH of the saliva is usually slightly on the acid side, about 6.8, although it may vary on either side of neutrality.

The longer food is retained in the mouth, the further this starch digestion proceeds and the more finely divided the food becomes as a result of chewing. In fact, even well-chewed food does not remain in the mouth long enough for much digestion of starch to take place, but saliva

continues to act for a time after the food has been swallowed. Relatively solid food takes about six seconds to pass from the mouth down the oesophagus to the stomach; liquid food travels more quickly.

Saliva keeps the mouth at a neutral pH and thus protects the teeth from decalcification and also keep the mouth and teeth clean.

Salivary Digestion

Saliva contains a starch-splitting enzyme, *salivary amylase* (*ptyalin*). Chloride ion activates this enzyme. Although saliva is capable of bringing about the hydrolysis of starch and glycogen to maltose, this is of little significance in the body because of the short time it can act on the food. Salivary amylase is readily inactivated at pH 4.0 or less, so that digestive action on food in the mouth will soon cease in the acid environment of the stomach. Furthermore, pancreatic amylase, which has a similar enzymatic action and specificity, is capable of accomplishing complete starch digestion. In many animals, a salivary amylase is entirely absent.

Digestion in the Stomach

This part of the alimentary canal has flexible walls and so can be extended as the food accumulates in it. This enables food from a particular meal to be stored for some time and released gradually to the rest of the alimentary canal.

Very little absorption takes place in the stomach except of ethanol ('alcohol') and certain drugs, but a glandular lining (Fig. 30.4) produces a digestive juice, *gastric juice* (the adjective *gastric* means of the stomach). Gastric juice contains the enzyme *pepsin*, and in young children it may also contain another enzyme called *rennin*. Pepsin acts on proteins and breaks them down into more soluble compounds called *peptides*, each made up of several amino acid units. Rennin, when present, clots the protein of milk. The stomach wall also secretes *hydrochloric acid* which makes a 0.5 per cent solution in the gastric Juice. The acid provides the most suitable degree of acidity (optimum pH) for pepsin to work in, and also kills many of the bacteria taken in with the food. The salivary amylase from the mouth cannot digest starch in such an acid medium, but it seems likely that it continues to act within the bolus of food until this is broken up and the hydrochloric acid reaches all its contents.

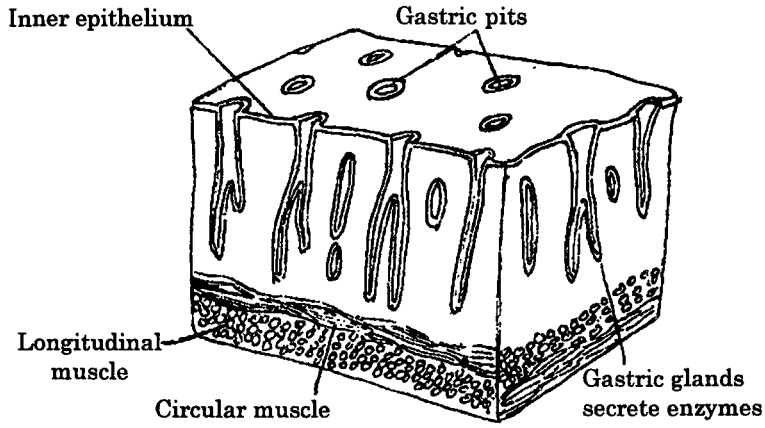


Fig. 30.4. Stereogram of section through stomach wall.

The rhythmic, peristaltic movements of the stomach, taking place about every twenty seconds, help to mix the food and gastric juice to a creamy fluid called *chyme*. Each wave of peristalsis also pumps a little of the chyme from the stomach into the first part of the small intestine, called

the *duodenum*. The pyloric sphincter is usually relaxed but contracts at the end of each wave of peristalsis, so limiting the amount of chyme which escapes. Even when relaxed, the pyloric opening is narrow and allows only liquid to pass through. When the acid contents of the stomach enter the duodenum, they set off a reflex action which closes the pyloric sphincter until the duodenal contents have been partially neutralised.

A meal of carbohydrate such as porridge may be retained in the stomach for less than an hour, and a mixed meal containing protein and fat for one or two hours.

Gastric Constituents and Gastric Digestion

In the mucosa of the stomach wall, 2 types of secretory glands are found : those exhibiting a single layer secreting cells (the chief cells) and those with cells arranged in layers (the parietal cells) which secrete directly into the gastric glands. The mixed secretion is known as *gastric juice*. It is normally a clear, pale yellow fluid of high acidity, 0.2-0.5% HCl, with a pH of about 1.0. The gastric juice is 97-99% water. The remainder consists of mucin and inorganic salts, the digestive enzymes (pepsin and rennin), and a lipase.

A. Hydrochloric acid : The parietal cells are the sole source of gastric hydrochloric acid. HCl originates according to the reactions shown in Fig. 30.4A.

The process is similar to that of the "chloride shift." There is also a resemblance to the renal tubular mechanisms for secretion of H^+, wherein the source of H^+ is also the *carbonic anhydrase*—catalyzed formation of H_2CO_3 from H_2O and CO_2. An alkaline urine often follows the ingestion of a meal ("alkaline tide"), as a result of the formation of bicarbonate in the process of hydrochloric acid secretion by the stomach in accordance with the reaction shown in Fig. 30.4A.

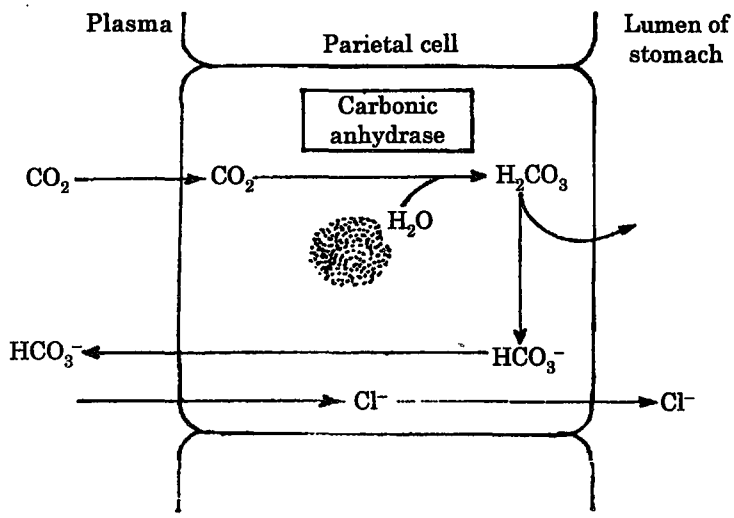


Fig. 30.4A. Production of gastric hydrochloric acid.

The various functions of hydrochloric acid are as follows :

1. It maintains the optimal pH (1.2—1.5) for digestion of proteins by pepsin.
2. It converts inactive pepsinogen into active pepsin.
3. It stimulates duodenum to liberate secretin.
4. It helps the absorption of iron by converting ferric hydroxide of the food into ferric form which is changed to the ferrous form by reduction.

5. It denatures food proteins making them more readily digestible.
6. It has a germicidal effect on micro-organisms and hence prevents the growth of micro-organisms in the stomach.

B. Pepsin : The chief digestive function of the stomach is the initiation of protein digestion. Pepsin is produced in the chief cells as the inactive zymogen, *pepsinogen.* This is activated to pepsin, first, by the proteolytic attack of another pepsinogen molecule at acid pH that splits off a polypeptide to expose active pepsin; and second, by pepsin, which rapidly activates further molecules of pepsinogen (*autocatalysis*). Pepsin has a molecular weight of 32700.

Pepsin transforms denatured protein into proteoses and then peptones, which are large polypeptide derivatives. Pepsin is an *endopeptidase,* since it hydrolyses peptide bonds within the main polypeptide structure rather than adjacent to N- or C-terminal residues, which is characteristic of *exopeptidases.* It is specific for peptide bonds formed by aromatic or dicarboxylic amino acids.

C. Rennin (Chymosin, Rennet) : The enzyme causes the coagulation of milk. This is important in the digestive processes of infants because it prevents the rapid passage of milk from the stomach. In the presence of calcium, rennin changes irreversibly the casein of milk to a paracasein which is then acted on by pepsin.

$$\text{Casein} \xrightarrow[Ca^{2+}]{\text{Rannin}} \text{calcium paracaseinate} + \text{peptides.}$$

Rennin is believed to be absent from the stomach of adults. It is used in making of cheese.

D. Lipase : Although it does contain a lipase capable of hydrolyzing triacylglycerols of short and medium chain length, the lipolytic action of gastric juice is not important.

Lipase can act only at the pH 5 to 7. Hence it is not so active at pH 1.3—1.5.

Digestion in Duodenum (Pancreatic and Intestinal Digestion)

The stomach contents, or *chyme,* which are of thick creamy consistency, are intermittently introduced during digestion into the duodenum through the pyloric valve.

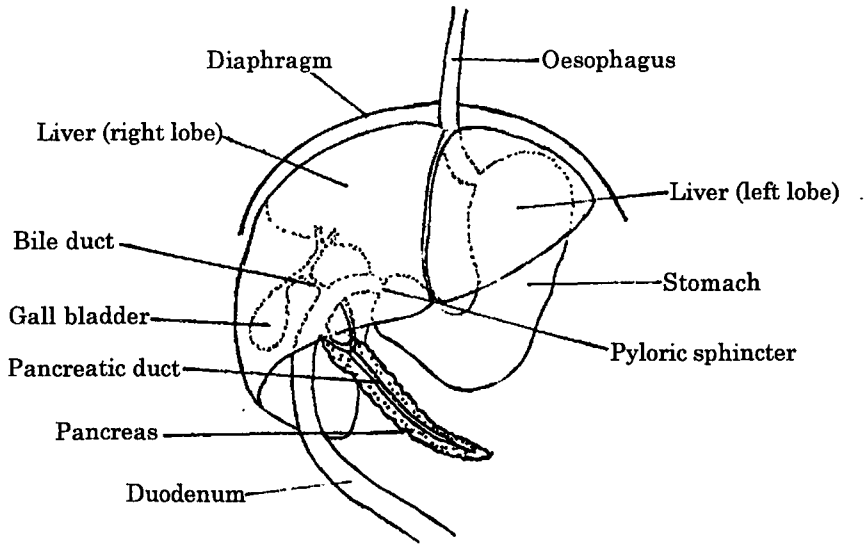


Fig. 30.5. Diagram to show relation of stomach, liver and pancreas.

Two alkaline fluids are poured into the chyme in the duodenum, *pancreatic juice* from the pancreas and bile from the liver. Both juices contain sodium hydrogen carbonate which partly

neutralizes the strongly acid chyme, so creating the slightly acid medium favourable to the action of the pancreatic and intestinal enzymes.

The *pancreas* ('sweetbread') is a cream-coloured gland lying below the stomach (Fig. 30.5). Its cells secrete several different enzymes. Three of these, including the powerful enzyme *trypsin*, break down proteins to peptides, and peptides to soluble amino acids. Others break down starch to maltose and fats to their constituent compounds, fatty acids and glycerol.

Bile is a green watery fluid made in the liver, stored in the gall bladder and conducted to the duodenum by the bile duct. Its colour is derived largely from breakdown products of the red pigment, hemoglobin, from decomposing blood cells. It contains sodium chloride, sodium hydrogencarbonate and complex organic compounds called *bile salts*, but no enzymes.

Bile dilutes the contents of the intestine, and the bile salts reduce the surface tension of fats, so emulsifying them, that is, dispersing them as tiny droplets presenting an increased surface for enzyme action and allowing more rapid digestion. Main of the bile salts are reabsorbed in the *ileum*, the lower part of the small intestine.

Bile Salts

In addition to many functions in intermediary metabolism, the liver, by producing bile, plays an important role in digestion. The gall-bladder, a saccular organ attached to the hepatic duct, stores a certain amount of the bile produced by the liver between meals. In humans, the gall bladder is a dispensable organ. During digestion, the gallbladder contracts and supplies bile rapidly to the small intestine by way of the common bile duct. The pancreatic secretions mix with the bile, since they empty into the common duct shortly before its entry into the duodenum.

Composition of Bile

The composition of hepatic bile differs from that of gall bladder bile. As shown in Table 30.1, the latter is more concentrated.

Table 30.1

THE COMPOSITION OF HEPATIC AND OF GALL BLADDER BILE

	Hepatic Bile (as secreted)		*Bladder Bile*
	Percent of Total Bile	*Percent of Total Solids*	*Percent of Total Bile*
Water	97.00	—	85.92
Solids	2.52	—	14.08
Bile acids	1.93	36.9	9.14
Mucin and pigments	0.53	21.3	2.98
Cholesterol	0.06	2.4	0.26
Esterified and non-esterified fatty acids	0.14	5.6	0.32
Inorganic salts	0.84	33.3	0.65
Specific gravity	1.01	—	1.04
pH	7.1-7.3	—	6.9-7.7

Bile Acids : In the liver, the synthesis of the primary bile acids takes place from cholesterol by several intermediate steps.

Cholic acid is the bile acid found in the largest amount in the bile itself. Both cholic acid and *chenodeoxycholic acid* are formed from a common precursor, itself derived from cholesterol.

The 7α-hydroxylation of cholesterol is the first committed step in the biosynthesis of bile acids, and it is probably this reaction that is rate-limiting in the pathway for synthesis of the acids. The α-hydroxylation reaction is catalyzed by a microsomal system; it requires oxygen and NADPH, and it is partially inhibited by carbon monoxide. This system appears similar to that for the mono-oxygenases previously described in connection with hydroxylation of steroids and of certain drugs. It appears that cytochrome P-450 is a component of the system, as it is for the 12 α-and 26-hydroxylation steps. Vitamin C deficiency interferes with bile acid formation at the 7 α-hydroxylation step and leads to cholesterol accumulation, hypercholesterolemia, and increased atherosclerosis in guinea pigs.

Under normal circumstances in humans, bile acids are synthesized by the liver at the relatively low rate of 200-500 mg/d. This rate is regulated to just replace the daily loss of bile acids in the feces. The bile acids are the end products of cholesterol catabolism in the body. Because the tissues cannot break down the steroid nucleus, these compounds, together with cholesterol itself, which is also present in the bile, represent the only significant route for *elimination of cholesterol from the body.* Measurement of the output of bile acids is therefore the most accurate way to estimate the amount of cholesterol lost from the body.

The bile acids normally enter the bile as glycine or taurine conjugates. The newly synthesized primary bile acids are considered to exist within the liver cell as esters of CoA, *i.e.,* cholyl-or chenodeoxycholyl-CoA (Fig. 30.6). The CoA derivatives are formed with the aid of an activating enzyme occurring in the microsomes of the liver. A second enzyme catalyzes conjugation of the activated bile acids (the CoA derivatives) with glycine or taurine to form glycoholic or glycochenodeoxycholic and taurocholic or taurochenodeoxycholic acids. These are the *primary bile acids.* In humans, the ratio of the glycine to the taurine conjugates is normally 3:1.

Since bile contains significant quantities of sodium and potassium and the pH is alkaline, it is assumed that the bile acids and their conjugates are actually in a salt form — hence the term "*bile salts.*"

Enterohepatic Circulation

A portion of the bile acids in the intestine undergoes changes by the activity of the intestinal bacteria. The deconjugation and 7 α-hydroxylation produce the secondary bile acids, deoxycholic acid from cholic acid and lithocholic acid from chenodeoxycholic acid. The conjugated and unconjugated bile salts are absorbed almost in the ileum. As fecal bile acids are present as the products of bacterial metabolism, therefore, it is assumed that metabolism within the intestinal lumen with reabsorption by passive diffusion is a component of the *enterohepatic circulation.* This mechanism helps to return 90% of the bile acids secreted into the intestine to the liver each day. But lithocholic acid is not reabsorbed to any significant extent due to its insolubility.

500 mg of bile salts per day are not absorbed and are eliminated in the feces. The enterohepatic circulation of bile salts is so efficient that a small amount of bile acids is cycled through the intestine 6-10 times a day with the loss of a small amount in the feces.

Functions of Bile

A. Emulsification : The bile salts have considerable ability to lower surface tension. This enables them to emulsify fats in the intestine and to dissolve fatty acids and water-insoluble soaps. The presence of bile in the intestine is an important adjunct to accomplish the digestion and absorption of fats as well as the absorption of the fat-soluble vitamins A, D, E, and K. When fat digestion is impaired, other foodstuffs are also poorly digested, since the fat covers the food particles and prevents enzymes from attaching them. Under these conditions, the activity of the intestinal bacterial causes considerable putrefaction and production of gas.

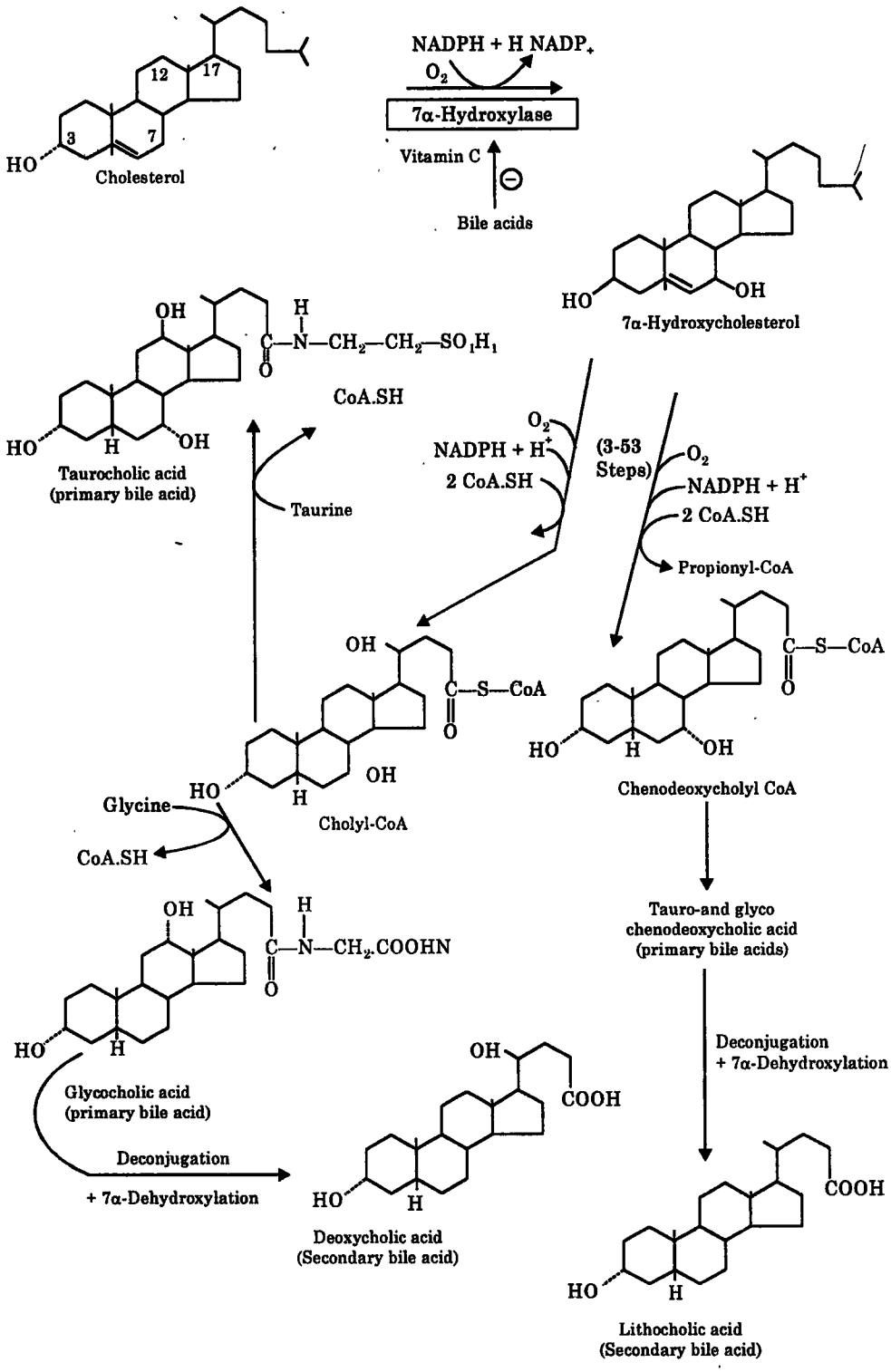


Fig. 30.6. Biosynthesis and degradation of bile acids.

B. Neutralization of Acid : In addition to its functions in digestion, the bile is a reservoir of alkali, which helps to neutralize the acid chyme from the stomach.

C. Excretion : As stated above, bile is an important vehicle for cholesterol excretion, but it also removes many drugs, toxins, bile pigments, and various inorganic substances such as copper, zinc and mercury.

D. Cholesterol Solubility in Bile; Formation of Gallstones : Free cholesterol is totally insoluble in an aqueous vehicle such as bile; consequently, it must be incorporated into a lecithin-

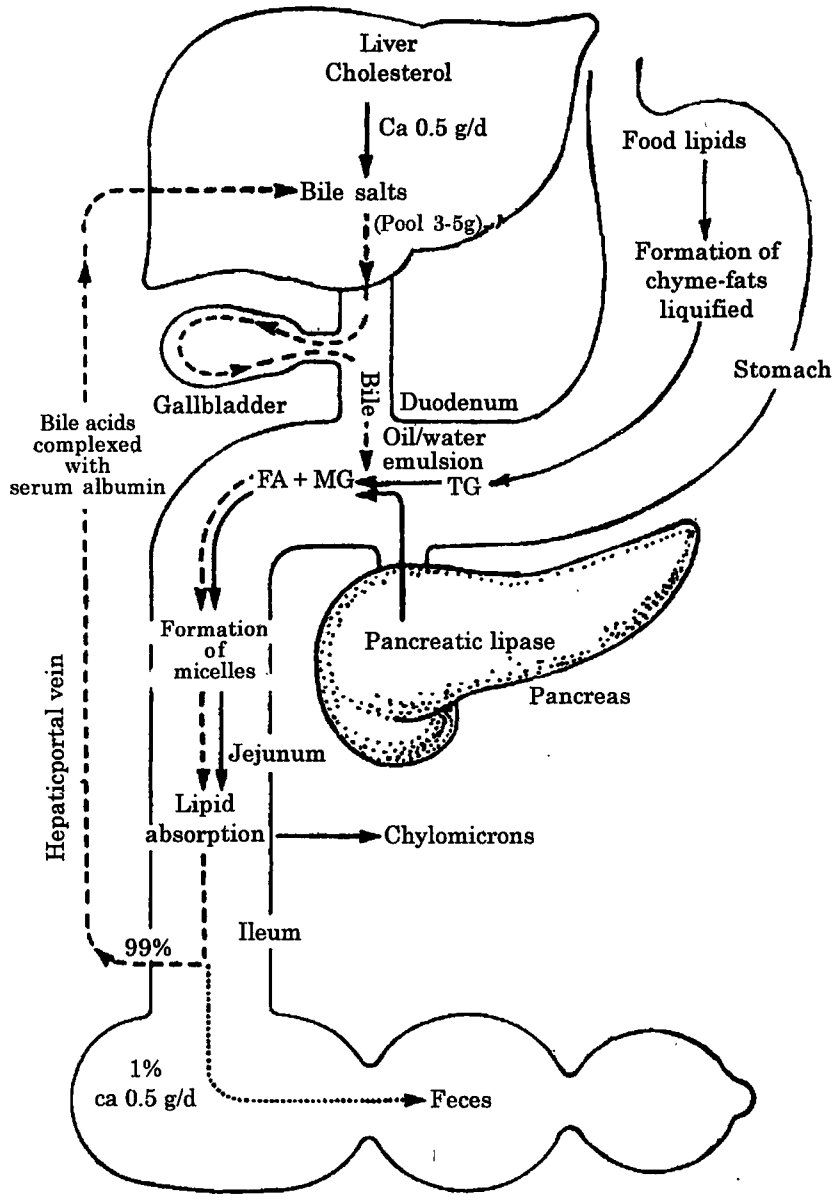


Fig. 30.7. Enterophepatic circulation of bile salts and the digestion of lipids. Dashes (.....) indicate enterohepatic, *c* circulation of bile salts. TG, triacylgycerol; MG, monoacylglycerol; FA, long chain fatty acids.

bile salt micelle. Indeed, lecithin, the predominant phospholipid in bile, is itself insoluble in aqueous systems but can be dissolved by bile salts in micelles. The large quantities of cholesterol present in the bile of humans are solubilized in these water-soluble mixed micelles, allowing cholesterol to be transported in bile via the biliary tract to the intestine. However, the actual solubility of cholesterol in bile depends on the relative proportions of bile salt, lecithin, and cholesterol. The solubility also depends on the water content of bile. This is especially important in dilute hepatic bile.

Gall stones are mainly of three types :

1. Cholesterol Stones

(*a*) These stones may be single or multiple.

(*b*) They may be white or yellowish.

(*c*) They may be mulberry shaped.

(*d*) They are not radio-opaque.

2. Pigment Stones

(*a*) These stones are formed by bile pigments, organic material and calcium.

(*b*) They are small multiple stones.

(*c*) They are hard, dark green or black.

(*d*) They are rarely radio-opaque.

3. Mixed Gall Stones

(*a*) These are composed of a mixture of cholesterol, bile pigments, protein and calcium.

(*b*) They are faceted dark brown stones with a hard shell and soft centre.

(*c*) They may be radio-opaque.

(*d*) They are the commonest forms of gall stones.

Constituents of Pancreatic Secretion

Pancreatic secretion is a non-viscid watery fluid that is similar to saliva in its content of water and contains some protein and other organic and inorganic compounds, mainly Na^+, K^+, HCO_3^-, and Cl^-, Ca^{2+}, Zn^{2+}, HPO_4^{2-}, and SO_4^{2-} are present in small amounts. The pH of pancreatic secretion is distinctly *alkaline,* 7.5-8.0 or higher.

Many enzymes are found in pancreatic secretion: some are secreted as zymogens.

A. Trypsin and Chymotrypsin : The proteolytic action of pancreatic secretion is due to the 2 endopeptidases trypsin and chymotrypsin, which attack protein, proteoses, and peptones from the stomach to produce polypeptides. Trypsin is specific for peptide bonds of basic amino acids whereas chymotrypsin is specific for peptides containing uncharged amino acid residues such as aromatic amino acids. Both enzymes are secreted as zymogens. Activation of *trypsinogen* is due to another proteolytic enzyme, *enterokinase,* secreted by the intestinal mucosa. Once trypsin is formed, it will attack the other zymogens in the pancreatic secretion, *chymotrypsinogen* and *procarboxypeptidase,* liberating chymotrypsin and *carboxypeptidase,* respectively.

B. Carboxypeptidase : The further attack on the polypeptides produced by the action of endopeptidases is carried on by the exopeptidase carboxypeptidase, which attacks the carboxyl terminal peptide bond, liberating single amino acids.

C. Amylase : The starch-splitting action of pancreatic secretion is due to a pancreatic α-amylase. It is similar in action to salivary amylase, hydrolyzing starch and glycogen to maltose, maltotriose, and a mixture of branched (1:6) oligosaccharides (α-limit dextrins) and some glucose.

D. Lipase : The pancreatic lipase acts at the oilwater interface of the finely emulsified lipid droplets formed by mechanical agitation in the gut in the presence of the bile salts, colipase (a protein present in pancreatic secretion), phospholipids, and *phospholipase* A_2 (also present in the pancreatic secretion). A limited hydrolysis of the ester bond in the 2 position of the phospholipid by phospholipase A_2 results in the binding of lipase to the substrate interface and a rapid rate of hydrolysis of triacylglycerol. The complete hydrolysis of triacylglycerols produces glycerol and fatty acids. However, the second and third fatty acids are hydrolyzed from the triacylglycerols with increasing difficulty. Pancreatic lipase is virtually specific for the hydrolysis of primary ester linkages, *i.e.,* at positions 1 and 3 of triacylglycerols.

Because of the difficulty of hydrolysis of the secondary ester linkage in the triacylglycerol, it is probable that the digestion of triacylglycerol proceeds by removal of the terminal fatty acids to produce 2-monoacylglycerol. Since this last fatty acid is linked by a secondary ester bond, its removal requires isomerization to a primary ester linkage. This is a relatively slow process as a result, 2-monoacylglycerols are the major end products of triacylglycerol digestion, and less than one-fourth of the ingested triacylglycerol is completely broken down to glycerol and fatty acids (Fig. 30.8).

E. Cholesteryl ester hydrolase (cholesterol esterase) : This enzyme may either catalyse the esterification of free cholesterol with fatty acids or, depending upon the conditions of equilibrium, it may catalyze the opposite reaction, *i.e.,* hydrolysis of cholesterol esters. According to Goodman, under the conditions existing within the lumen of the intestine, the enzyme catalyzes the hydrolysis of cholesterol esters, which are thus absorbed from the intestine in a nonesterified, free form.

F. Ribonuclease (RNase) and deoxyribonuclease (DNase) : have been prepared from pancreatic tissue.

These enzymes are specific for the hydrolysis of RNA and DNA respectively. These are endonucleases. Both the enzymes are capable of cleaving internal phosphodiester bonds of produce a 3′-hydroxyl and a 5′-phosphoryl or a 5′-hydroxyl and a 3′-phosphoryl terminus. Some are capable of hydrolyzing both strands of a double stranded molecule whereas others can only cleave single strands of nucleic acids. Some nucleases are exonucleases. These are capable of hydrolyzing a nucleotide when it is present at a terminus of a molecule.

G. Phospholipase A_2 *:* Phospholipase A_2 hydrolyzes the ester bond in the 2 position of glycerophospholipids of both biliary and dietary origins to lysophospholipids.

H. Aminopeptidase and dipeptidase : The aminopeptidase attacks the terminal peptide bond at the free amino end of the chain.

I. Cholesterol ester hydrolase (cholesterol esterase) : This enzyme hydrolyses the esterification of free cholesterol with fatty acids.

J. Collagenase : This enzyme hydrolyses collagen present in meat and fish.

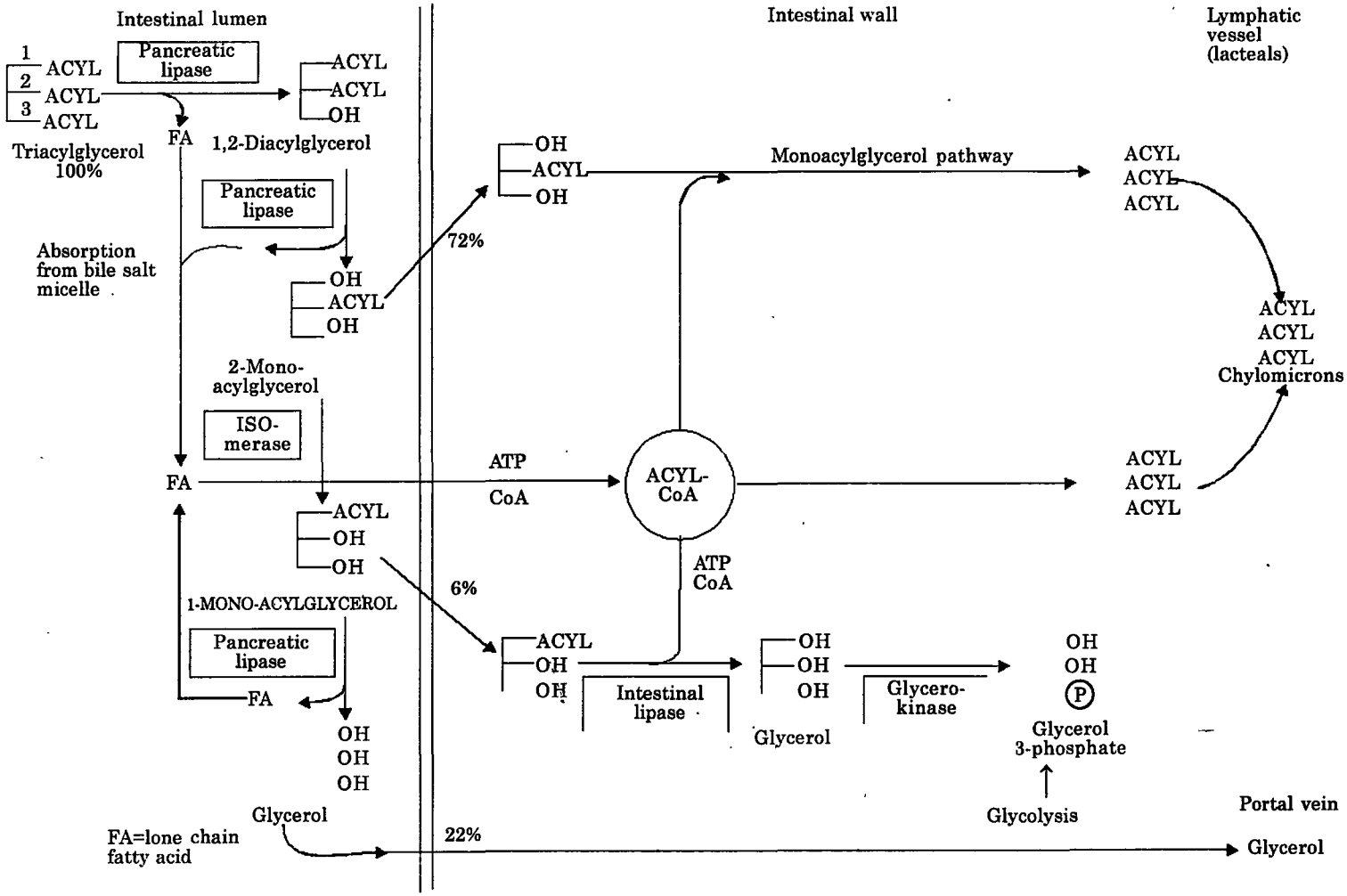


Fig. 30.8. Chemical mechanisms of digestion and absorption of triacylglycerols. FA, long chain fatty acid.

Constituents of Intestinal Secretion

The intestinal juice secreted by the glands of Brunner and of Lieberkuhn also contains digestive enzymes, including the following :

(1) *Aminopeptidase,* is an exopeptidase attacking peptide bonds next to N-terminal amino acids of polypeptides and oligopeptides, and *dipeptidases* of various specificity, some of which may be within the intestinal epithelium. The latter completes digestion of dipeptides to free amino acids.

(2) Specific *disaccharides* and *ligosacharidases, i.e., α-glucosidase* (*maltase*), remove single glucose residues from α (1-4) linked oligosaccharides and disaccharides, *α-dextrinase,* which hydrolyze 1 → 6 bonds in α-limit dextrins, *β-galactosidase* (lactase) for removing galactose from lactose, and *sucrase* for hydrolyzing sucrose.

(3) A *phosphatase,* removes phosphate from certain organic phosphates such as hexosephosphates, glycerophosphate, and the nucleotides derived from the diet and the digestion of nucleic acids by nucleases.

(4) *Polynucleotidases,* split nucleic acids into nucleotides.

(5) *Nucleosidases* (nucleoside phosphorylases), one of which attacks only guanine—and hypoxanthine—containing nucleosides. The pyrimidine nucleosides (uridine, cytidine, and thymidine) are broken down by another enzyme that differs from the purine nucleoside phosphorylase.

(6) The intestinal secretion is also said to contain a *phospholipase* that attacks phospholipids to produce glycerol, fatty acids, phosphoric acid, and bases such as choline.

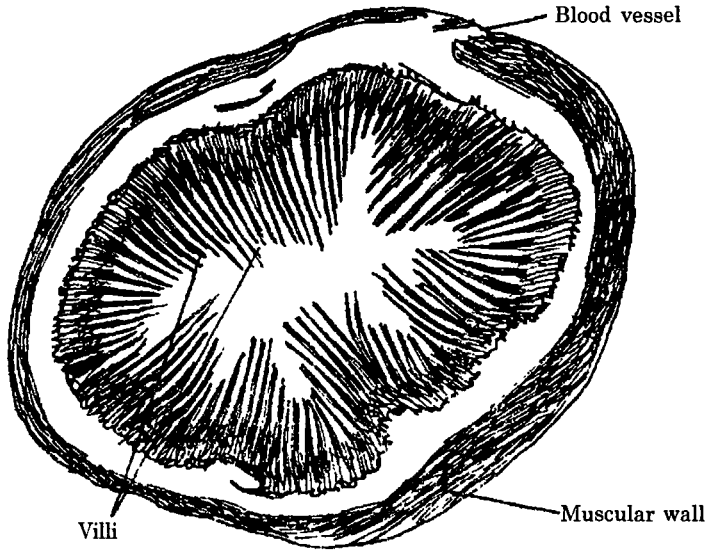


Fig. 30.9. Transverse section through ileum of cat, showing villi (× 10).

Digestion in the Ileum

The glands in the lining of the ileum (Fig. 30.9) produce mucus but no appreciable amount of digestive enzymes. The pancreatic enzymes, however, complete the breakdown to amino acids

of any peptides still undigested of maltose and other carbohydrates to glucose and related sugars like fructose and galactose, and of unchanged fats to fatty acids and glycerol. These products are all-water-soluble and so can pass through the intestinal lining and into the bloodstream.

Although the action of digestive enzymes dissolves all digestible food constituents, the cells which produce them are not themselves attacked by the enzymes, nor are the other cells lining the alimentary canal. This is partly because the enzymes are produced within the cells in an inactive form and cannot work until they are secreted into the cavity of the alimentary canal, where they are activated by the chemicals present. Pepsin, for example, is made by the glands of the stomach lining in the form of an inactive substance (or *precursor*) called *pepsinogen;* pepsinogen is only converted into the active enzyme when it is secreted into the stomach and comes into contact with the hydrochloric acid in the gastric juice. The alimentary canal is additionally protected from enzyme attack by the constant production of *mucus* by specialised cells in the epithelium. Mucus coats the epithelium with a slimy, viscous layer which helps to prevent the digestive juices from reaching it; it also lubricates the walls of the canal, and assists the passage of food along it.

Major Products of Digestion

The final result of the action of the digestive enzyme described is to reduce the foodstuffs of the diet to forms that can be absorbed and assimilated. These end products of digestion are, for carbohydrates. the monosaccharides (principally glucose); for proteins, the amino acids; for triacylglycerol, the fatty acids, glycerol, and monoacylglycerol, and for nucleic acids, the nucleobases, nucleosides, and pentoses.

Absorption in the Illeum

The ileum is an organ of absorption rather than an organ of digestion; nearly all the products of digestion are absorbed into the bloodstream through its walls. Certain of its characteristics are important adaptations to its absorbing function :

(*a*) in most species it is fairly long (several metres in man), presenting a large absorbing surface to the digested food;

(*b*) its internal surface is enormously increased by a covering of thousands of tiny finger-like projections called *villi,* each a millimetre or so long (Figs. 30.9 and 30.10), rather like the pile of a fabric like velvet;

(*c*) the epithelium covering the villi is very thin, and fluids can pass through it fairly easily;

(*d*) each villus contains a dense network of minute thin-walled blood vessels or *capillaries* (Fig. 30.11).

The small molecules of the digested food, principally amino acids and glucose, pass through the epithelium and the capillary walls; the mechanism of their movement involves both simple diffusion and some kind of 'active transport.' They are then carried away in the capillaries, which unite to form small veins the eventually join up to form one large vein, the *hepatic portal vein.* This carries all the blood from the intestine to the liver, which may retain or alter any of the digestion products. The digested food is then carried in the blood-stream to all parts of the body.

Some of the fatty acids and glycerol from the digestion of fats enter the blood capillaries of the villi, but a large proportion recombine in the intestinal lining to form fats again. These fats pass into vessels in the villi called *lacteals* which derive their name from their milky-white appearance (the Latin word for milk is *lac*) due to the minute droplets of fat in the fluid they contain. Some of this fat may actually pass through the epithelium into the lacteals in finely emulsified form without any prior digestion.

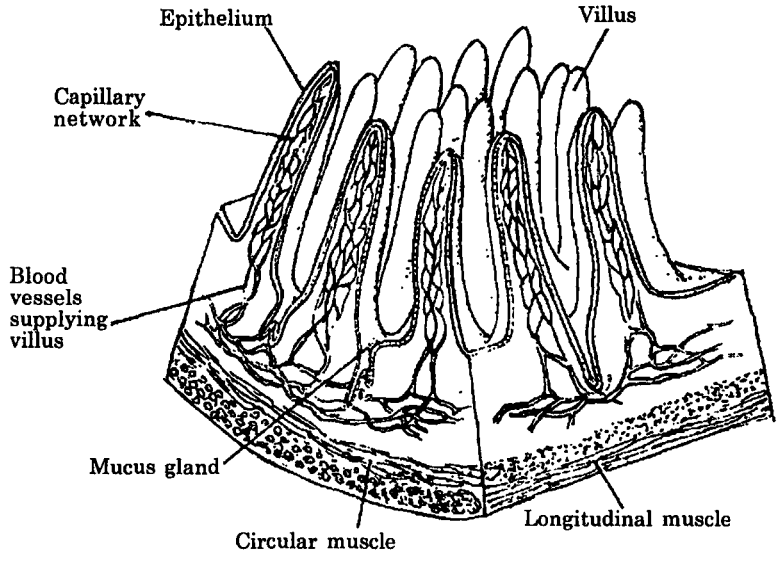


Fig. 30.10. Stereogram to show structure of ileum.

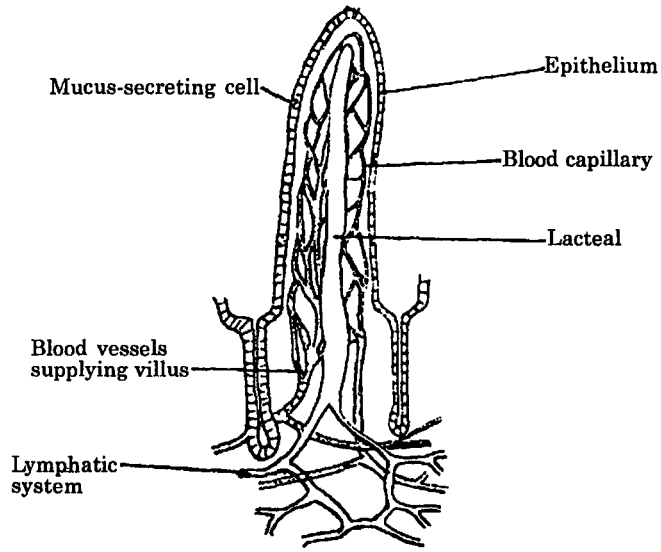


Fig. 30.11. Villus structure.

The fluid in the lacteals drains into *lymph vessels* which form a network all over the body called the *lymphatic system;* its contents are eventually emptied into the bloodstream.

Caecum and Appendix

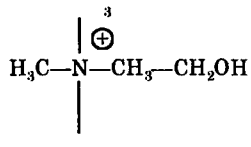
In many animals the *caecum* forms a branch of the alimentary canal at the point where the ileum meets the large intestine; it ends blindly in the *appendix.* In herbivorous animals like the rabbit and the horse these organs are quite large, and it is here that most of the digestion of cellulose takes place, largely as a result of bacterial activity. In man the caecum and the appendix are relatively small, and are probably vestigial structures, that is, organs which have apparently lost their functions through disuse in the course of evolution.

Large Intestine

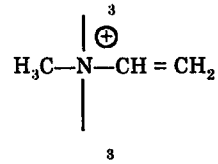
The large intestine consists of two parts, the *colon* and the *rectum*. The material which passes from the ileum into the colon consists of water with undigested matter, largely cellulose and vegetable fibres (the 'roughage'), bacteria, mucus and lead cells from the lining of the alimentary canal. The large intestine secretes no enzymes, but the bacteria in the colon digest part of the fibre. Much of the water from the undigested residues is absorbed in the colon. The semi-solid waste, the *faeces*, passes into the rectum by peristalsis and is expelled at intervals through the *anus*. The residues may spend 12 to 24 hours or more in the intestine, depending on the proportion of fibre in the diet.

Intestinal Putrefaction and Fermentation

Most ingested food is absorbed from the small intestine. The residue passes into the large intestine. Here considerable absorption of water takes place, and the semiliquid intestinal contents gradually become more solid. During this period, considerable bacterial activity occurs. By fermentation and putrefaction, the bacteria produce various gases, such as CO_2, methane, hydrogen, nitrogen, and hydrogen sulfide, as well as acetic, lactic, and butyric acids. The bacterial decomposition of lecithin may produce choline and related toxic amines such as neurine.



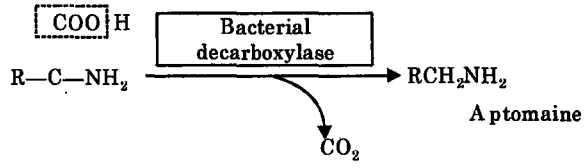
Choline



Neurine

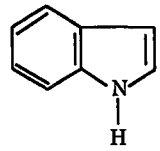
Fate of Amino Acids

Many amino acids undergo decarboxylation as a result of the action of intestinal bacteria to produce toxic amines (ptomaines)

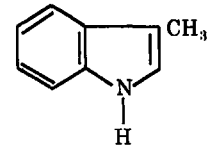


Such decarboxylation reactions produce cadaverine from lysine; agmatine from arginine; tyramine from tyrosine; putrescine from ornithine; and histamine from histidine. Many of these amines are powerful vasopressor substances.

The amino acid tryptophan undergoes a series of reactions to form indole methylindole (skatole), the substances particularly responsible for the odor of feces.

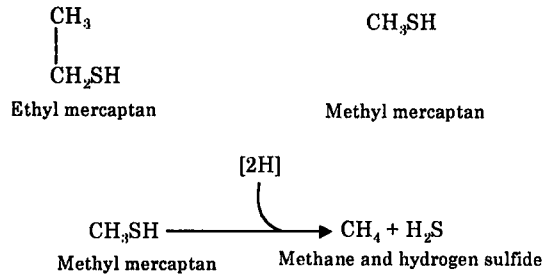


Indole



Skatole

The sulfur-containing amino acid cysteine undergoes a series of transformations to form mercaptans such as ethyl and methyl mercaptan as well as H_2S.



The large intestine is a source of considerable quantities of ammonia, presumably as a product of the putrefactive activity on nitrogenous substrates by the intestinal bacteria. This ammonia is absorbed into the portal circulation, but under normal conditions it is rapidly removed from the blood by the liver. In liver disease this function of the liver may be impaired, in which case the concentration of ammonia in the peripheral blood will rise to toxic levels. It is believed that ammonia intoxication may play a role in the genesis of hepatic coma in some patients. In dogs on whom an Eck fistula has been performed (complete diversion of the portal blood to the vena cava), the feeding of large quantities of raw meat will induce symptoms of ammonia intoxication (meat intoxication) accompanied by elevated levels of ammonia in the blood. The oral administration of neomycin has been shown to reduce the quantity of ammonia delivered from the intestine to the blood, due undoubtedly to the antibacterial action of the drug. The feeding of high-protein diets to patients suffering from advanced liver disease, or the occurrence of gastrointestinal hemorrhage in such patients, may contribute to the development of ammonia intoxication. Neomycin is also beneficial under these circumstances.

Intestinal Bacteria

The intestinal flora may comprise of as much as 25% of the dry weight of the feces. In herbivora, whose diet consists largely of cellulose, the intestinal or ruminal bacteria are essential to digestion, since they decompose the polysaccharide and make it available for absorption. In addition, these symbiotic bacteria accomplish the synthesis of essential amino acids and vitamins. In humans, although the intestinal flora is not as important as in herbivora, nevertheless some nutritional benefit is derived from bacterial activity in the synthesis of certain vitamins, particularly vitamins K and B_{12}, and possibly other members of the B complex, which are made available to the body. Information gained from experiments with animals raised under strictly aseptic conditions should help to define further the precise role of the intestinal bacteria.

30.5 How Digested Food is Used

The products of digestion are carried round the body in the blood from which they are absorbed and metabolized by the cells.

(a) Glucose is oxidized during respiration in the protoplasm. This reaction releases energy to drive the many chemical processes in the cell and in specialised cells to produce, for example, contraction (muscle cells) and electrical changes (nerve cells).

(b) Fats are incorporated into cell membranes and other structures in cells and fatty acids are oxidized in muscles to provide energy for muscle contraction. More than twice as much energy is obtained in this way from a given weight of fat as from the same amount of glucose.

(c) Amino acids are reassembled in the cells to make proteins; these proteins may go to make up the cells membrane, the nucleus, the cytoplasm or the structures contained in it, or they may be enzymes controlling and co-ordinating the chemical activity within the cell or secreted by it for the control of extracellular reactions.

30.6 Storage of Digested Food

If the quantity of food taken in exceeds the body's needs for energy or for structural materials, the surplus must be stored.

(a) Glucose (Fig. 30.12) *:* The concentration of glucose in the blood of a person who has not eaten for eight hours is usually between 90 and 100 mg per 100 cm^3 of blood. After a meal containing carbohydrate, the blood glucose level may rise to 140 mg per 100 cm^3, but within two hours the level returns to about 95 mg per 100 cm^3. Some of the glucose will have been oxidized to supply energy; the rest would have been removed from the blood as it passes through the liver, and converted into *glycogen*, the molecule of which consists of a long branching chain of glucose units, rather like that of starch. Glycogen is insoluble in water; about 100 g of the compound is stored in the liver and 300 g in the muscles. When the blood glucose level falls below about 80 mg per 100 cm^3, the glycogen in the liver is converted back into glucose which is released into the blood. The muscle glycogen, however, it not normally returned to the blood circulation but is reconverted to glucose for use as an energy source within the muscles themselves.

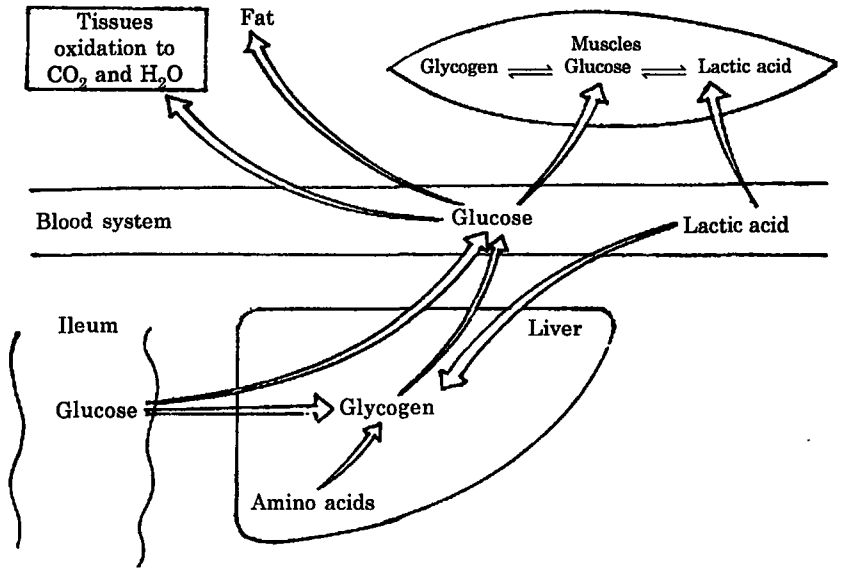


Fig. 30.12. Carbohydrate metabolism.

If the energy demand is high—for example, during vigorous exercise—insufficient oxygen may be available for complete oxidation of glucose to carbon dioxide and water. Lactic acid, the product of anaerobic respiration, may then accumulate in the muscle. It is carried away from the muscle in the blood and returned to the liver, where it may again be converted into glycogen, and stored.

The glycogen in the liver is a 'short-term' store; if no other glucose supply is available it will last the body for about six hours. Excess glucose not stored as glycogen is converted into fat and stored in fat cells (see below).

(b) Fats : Certain cells can accumulate drops of fat in their cytoplasm. As these drops increase in size and number, they join together to form one large globule of fat in the middle of the cell, pushing the cytoplasm into a thin layer and the nucleus to one side (Figs. 30.13 and 30.14). Groups of fat cells form *adipose tissue* beneath the skin and in the connective tissue of most organs.

Unlike glycogen, there is no limit to the amount of fact that can be stored in the body, and because of its high energy value it is an important food reserve.

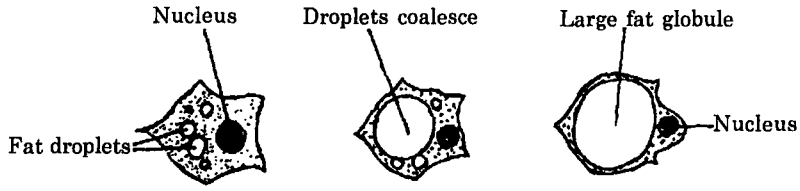


Fig. 30.13. Accumulation of fat in a fat cell.

(c) Amino acids (Fig. 30.15) *:* Amino acids are not stored in the body. Those not used in protein formation are deaminated. The protein of the liver and tissues can act as a kind of protein store to maintain the protein level in the blood but absence of protein in the diet soon leads to serious disorders.

Overweight

When the energy value of an individual's food intake exceeds his body's energy requirements, the excess food is stored mainly as fat : putting on weight is unquestionably the result of eating more food than the body needs. But it is still not fully understood why some people find it difficult to restrict their intake of food to the amount required to meet their needs, not why some never seem to get fat however much they eat while others put on weight even when their intake only marginally exceeds their requirements. A partial explanation of people's differing reactions probably lies in the balance between the relative amounts of the various hormones produced by their bodies, a balance which is to some extent determined by hereditary factors.

An obese person can lose weight by eating food which supplies him with less energy than his body needs so that his energy requirements must be met in part from his fat reserves. A 'slimming diet' designed to reduce energy intake must, always include sufficient amounts of the essential amino acids, vitamins, minerals and certain essential fatty acids.

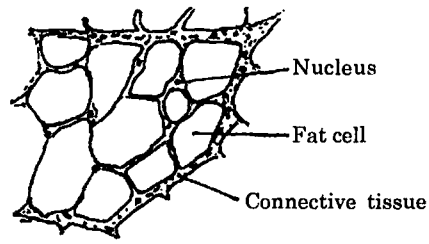


Fig. 30.14. Small section of adipose tissue.

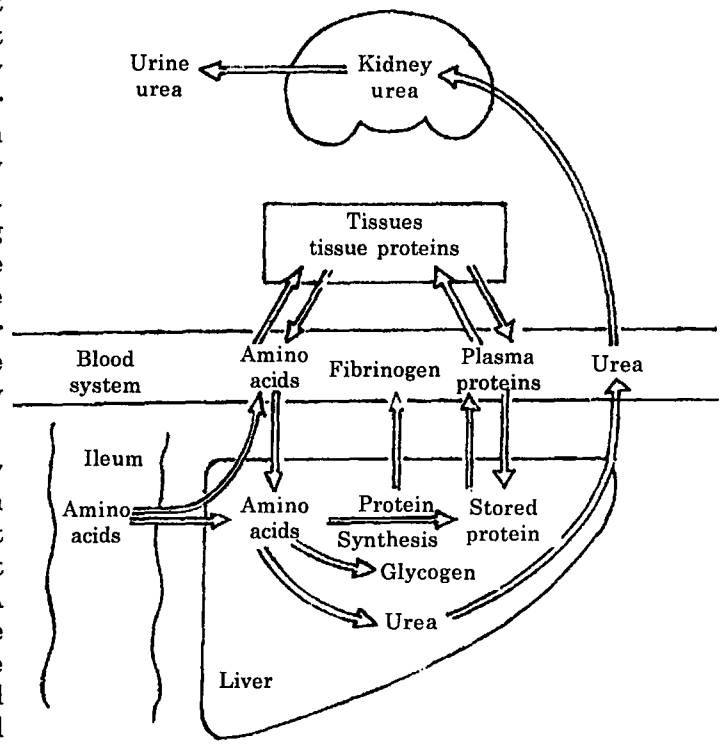


Fig. 30.15. Protein metabolism.

30.7 Liver

The liver is a large, reddish-brown organ lying just below the diaphragm and partly overlapping the stomach. It is supplied with oxygen by blood carried to it in the *hepatic artery* (*hepatic* means 'of the liver'); in addition it receives all the blood which leaves the walls of the alimentary canal in the *hepatic portal vein.* It has a great many important functions.

(a) Regulation of blood glucose levels : Glucose enters the blood from the digested food in the ileum. If there is too much glucose in the blood some of it is removed by converting it into insoluble glycogen in the liver. It, on the other hand, the glucose level falls too low, some of the glycogen in the liver is converted by enzyme action into glucose which enters the blood and replenishes the supply. If the concentration of glucose rises above 160 mg per 100 cm^3 of blood, glucose is excreted by the kidneys in urine; if it falls below 40 mg per 100 cm^3, the brain cells may be affected by glucose deficiency, leading to convulsions, and unconsciousness. By helping to keep the glucose concentration between 80 and 150 mg per 100 cm^3 the liver prevents these undesirable effects.

(b) Formation of bile : Bile is produced continuously by liver cells, but it is stored and concentrated in the gall bladder; it is poured into the duodenum when the acid chyme is discharged into it from the stomach. It contains *bile salts* produced by the liver which are important in the emulsification and subsequent absorption of fats; they are reabsorbed with the fats they emulsify and eventually return in the bloodstream to the liver. Bile owes its colour to the green and yellow pigments formed in the daily breakdown of millions of red blood cells; the pigments are removed from the blood by the liver and excreted into the duodenum in the bile, ultimately leaving the body in the faeces.

(c) Storage of iron : The breakdown of red blood cells is completed in the liver. Their red pigment, haemoglobin, is a protein which contains iron; the iron from haemoglobin decomposition is stored in the liver and eventually incorporated into new red blood cells.

(d) Deamination : Amino acids over and above the body's needs are not stored as such. Those which are not built up into proteins for the manufacture of new or replacement protoplasm or for enzyme production are converted into carbohydrates by *deamination,* that is, the removal of the nitrogen-containing amino group—NH_2, from their molecules. The nitrogen of the amino group is converted in the liver into the soluble compound *urea.* $NH_2.CO.NH_2$, which is carried in the bloodstream to the kidneys where it is removed from the blood and excreted in the urine. The carbohydrate residue is converted into glycogen and either stored or oxidized to release energy.

(e) Manufacture of plasma proteins : The fluid part of the blood, the *blood plasma,* contains a number of proteins in solution. The liver makes most of these proteins, including fibrinogen, which plays an important part in the clotting of blood.

(f) Metabolism of fats : When the fats stored in the body are needed for the supply of energy, they are carried in the blood from the fat depots. Some are used directly by the muscles and some are converted by the liver to substances which can be oxidized for energy by other tissues.

(g) Maintenance of body temperature : Chemical changes, including those listed above and a great many others, are constantly going on in the liver; many of these release energy in the form of heat. This heat is carried throughout the body by the blood and helps to maintain body temperature.

(h) Detoxication : The cation of the bacteria on amino acids in the large intestine produces certain poisonous substances which are absorbed by the blood, but which on reaching the liver are converted into harmless compounds that can be safely carried in the bloodstream to the kidneys and are excreted in the urine. Many other substances are similarly modified by the liver before

being excreted. Hormones, made by the body itself, and certain drugs are converted into inactive compounds in the liver, so limiting the period during which they can influence the body's metabolism.

(i) Storage of vitamins : The fat-soluble vitamins A and D are stored in the liver. This is the reason why liver, especially fish liver, is a valuable source of these vitamins in the diet. The liver also stores a product of the vitamin B_{12} which is necessary for the normal production of red cells in the bone marrow.

30.8 Homeostasis

If a mobile, single-called animal such as *Amoeba* or *Paramecium* finds itself in conditions which are unfavourable—for example, too acidic, too warm or too light — it is capable of moving until it encounters more suitable conditions.

The cells in a multicellular organism cannot move to a fresh environment but are not less dependent on a suitable temperature and pH for the chemical reactions which maintain life. It is therefore essential to their efficient functioning that the medium round them does not alter its composition very much. In fact, the composition and concentration of the body fluids remain remarkably constant, with only minor fluctuations. Even though the food eaten may vary from day-to-day in its amount and chemical nature, the composition of the blood, and of the fluid which bathes all the cells of the body, changes comparatively little. This constancy of the *internal environment,* as it is called, is of great importance to the smooth functioning of the cells of the body. If it were not maintained, the chemical changes in their protoplasm would become so erratic and unpredictable that quite a slight change of diet or activity might bring about a complete breakdown of the body's biochemistry. Enzymes, for example, can only function properly within a certain narrow range of acidity or alkalinity; if the acidity of the body's fluids were allowed to rise beyond normal levels, the activity of essential enzymes would be inhibited and life could not continue. A rise in the concentration of the body fluids might produce a disastrous withdrawal of water from the cells by osmosis; a fall in body temperature could slow down vital chemical reactions.

The regulation of the internal environment is called *homeostasis.* The liver pays a vital part in the process, in the several ways in which it maintains the composition of the blood.

Table 30.2

SUMMARY OF DIGESTIVE PROCESSES

Source of Secretion and Stimulus for Secretion	*Enzyme*	*Method of Activation and Optimal Conditions for Activity*	*Substrate*	*End Products or Action*
1	*2*	*3*	*4*	*5*
Salivary glands of mouth : Secrete saliva in reflex response to presence of food in mouth.	Salivary amylase	Chloride ion necessary pH 6.6-6.8	Starch Glycogen	Maltose plus 1:6 glucosides (oligosaccharides) plus of food in mouth.maltotriose
Stomach glands : Chief cells and parietal cells secrete gastric juice in response to reflex stimulation and chemical action of gastrin.	Pepsin	Pepsinogen converted to active pepsin by HCl pH 1.0-2.0	Protein	Proteoses Peptones
	Rennin	Calcium necessary for activity. pH 4.0.	Casein of milk	Coagulates milk
Pancreas : Presence of acid chyme from the stomach activates duodenum to produce (1) secretion which hormonally stimulates flow of pancreatic juice; (2) cholecystokinin, which stimulates the production of enzymes.	Trypsin	Trypsinogen converted to active trypsin by enterokinate of intestine at pH 5.2-6.0. Autocatalytic at pH 7.9.	Protein Proteoses Peptones	Polypeptides Dipeptides
	Chymotrypsin	Secreted as chymotrypsinogen and converted to active form by trypsin. pH 8.0	Protein Proteoses Peptones	Same as trypsin. More coagulating power for milk.
	Carboxypeptidase	Secreted as procarboxy-peptidase, activated by trypsin.	Polypeptides at the free carboxyl end of the chain	Lower peptides. Free amino acids.
	Pancreatic amylase	pH 7.1	Starch Glycogen	Maltose plus 1 : 6 glucosides (oligosaccharides) plus maltotriose.
	Lipase	Activated by bile salts, phospholipids, colipase, pH 8.0	Primary ester linkages of triacylglycerol	Fatty cids, monoacylglycerols, diacylglycerols, glycerol
	Ribonuclease		Ribonucleic acids	Nucleotides
	Deoxyribonuclease		Deoxyribonucleic acids	Nucleotides
	Cholesteryl ester hydrolase	Activated by bile salts.	Cholesteryl esters	Free cholesterol plus fatty acids
	Phospholipase A_2		Phospholipids	Fatty acids, lysophospholipids.

(Contd.)

1	2	3	4	5
Liver and gallbladder : Cholecsytokinin, a hormone from the intestinal mucosa—and possibly also gastrin and secretin—stimulate the gallbladder and secretion of bile by the liver.	(Bile salts and alkali)		Fats also neutralize acid chyme	Fatty acid—bile salt conjugates and finely emulsified neutral fat—bile salt micelles
Small intestine : Secretions of Brunner's glands of the duodenum and glands of Lieberkuhn.	Aminopeptidase		Polypeptides at the free amino end of the chain	Lower peptides. Free (amino acids)
	Dipeptidases		Dipeptides	Amino acids
	Sucrase	pH 5.0-7.0	Sucrose	Fructose, glucose
	Maltase	pH 5.8-6.2	Maltose	Glucose
	Lactase	pH 5.4-6.0	Lactose	Glucose, glactose
	Phosphatase	pH 8.6	Organic phosphates	Free phosphate
	Isomaltase or 1:6 glucosidase		1:6 glucosides	Glucose
	Polynucleotidases		Nucleic acids	Nucleotides
	Nucleosidases (nucleoside phosphorylases)		Purine or pyrimidine nucleosides	Purine or pyrimidine bases, pentose phosphate.

31 Biochemistry of Respiration

31.1 Introduction

Respiration may be defined as the series of chemical changes which release energy from food material in both plants and animals. The energy so produced is used for the wide range of activities which characterise for living things. These may include muscular contraction, conduction of impulses along nerves and driving of the cell reactions which are part of the vital chemistry of life and which may lead to the build up of new wills or the maintenance of older ones or to the secretions of products such as the digestive juices of animals or nectar of the flowers. Every stage of the complex chain of reactions involved in respiration is more or less controlled by biological catalyst called enzyme. An enzyme controls the rate of a chemical reaction in living material and generally that of one particular reaction.

A distinction is usually made between two forms of, or stages in, respiration. ***Aerobic respiration*** involves the release of energy by the reaction of oxygen with carbohydrates which are completely broken down to carbon dioxide and water. ***Anaerobic respiration***, on the other hand, releases energy from carbohydrates by reactions which do not involve oxygen from the air.

An average adult man, who at least, utilises about 250 ml of oxygen and eliminates 200 ml of carbon dioxide per minute. However, these quantities become ten times during strenuous exercise. The supply of the required amount of oxygen to the tissues and the elimination of CO_2 have been brought about by blood. The arterial blood supplies required oxygen to the tissues and takes up carbon dioxide from them and gets converted to venous blood. In turn, this enters the lungs where it gives up carbon dioxide and takes up oxygen from alveolar air to get reconverted in to arterial blood. Table 31.1 includes the average gaseous composition of arterial and venous blood.

Table 31.1

	O_2		CO_2		N_2	
	ml/100 ml.	pO_2 *mm.Hg.*	*ml/100 ml.*	pCO_2 *mm.Hg.*	*ml/100 ml.*	pN_2 *mm.Hg.*
Inspired air	20.9	158	0.04	0.3	79.0	597
Alveolar air	14.2	100	5.6	40	80.3	570
Arterial blood	19.6	100	48.2	40	0.9	570
Venous blood	12.6	40	54.8	46	0.9	570
Interstitial fluid		30				
Tissue fluid		10		50		

The aqueous tension in alveolar air is about 48 mm Hg.

The compositions of atmospheric and expired air are given as follows :.

	Atmospheric air (Inspired air)	*Expired air*
Oxygen	20.96%	15%
Carbon dioxide	0.04%	5%
Nitrogen	79%	79%

About 1/4 of the oxygen of the inspired air has passed into the blood and has been replaced in the expired air by equal amount of CO_2.

Partial Pressure of Gases

The atmospheric air contains nitrogen, oxygen, carbon dioxide and water vapour. The pressure exerted by a mixture of gases is equal to the sum of their partial pressures.

$$AP–PH_2O = P_{N2} + PCO_2 + PO_2$$

The partial pressure of the gases (Y) N_2, O_2 and CO_2 can be calculated as follows :

$$Py = \frac{(760 - P_{H_2O})X\%Y}{100}$$

31.2 Transport of Oxygen by Blood

When the venous blood enters the lungs, it contains 12.6 ml oxygen at a partial pressure (conventionally referred to as pO_2) of 40 mm. of Hg. It gets separated from the alveolar air by only a thin endothelial lining of the capillaries and alveoli (not more than 1-2 μ thick). The alveolar air is having oxygen at a pO_2 of 100 mm. This makes oxygen to rapidly diffuse through the thin membrane into the blood, first in physical solution into plasma, later to be taken up by the hemoglobin of the erythrocyte. The total surface of the alveoli happens to be so large (50 to 100 sq. meters) that during the short period of about 0.7 second the blood takes to traverse the lung capillaries, it attains equilibrium with alveolar air and becomes the arterial blood.

Arterial blood is having an average of 19.6 ml/100 ml at a pO_2 of 90 mm of Hg. At this pressure only 0.3 ml of oxygen is in physical solution while the rest is present in loose combination with hemoglobin as oxyhemoglobin.

$$Hb + O_2 \longleftrightarrow HbO_2$$

Hemoglobin is 97% saturated with oxygen under these conditions. The arterial blood now passes to the tissues and as it enters the capillary bed of the tissues, it again gets separated only

by a thin endothelial lining of the capillaries from the interstitial fluid, where the pO_2 is only 30 mm. The O_2 in physical solution rapidly diffuses out first and followed by O_2 released by the dissociation of HbO_2.

$$HbO_2 \longleftrightarrow Hb + O_2$$

By the time the blood traverses the capillary bed and becomes venous blood, it has lost about 7.0 ml of oxygen and the pO_2 decreases to 40 mm. The hemoglobin is only 70% oxygenated.

These changes involved in oxygen transport are closely interconnected with changes which are involved in CO_2 transport and resulting pH changes in blood.

Oxygen Dissociation Curves

If a solution of myoglobin (which is having only one heme per molecule) is exposed to pure oxygen and the per cent saturation of the solution with oxygen is measured, the relationship between oxygen tension and per cent saturation of myoglobin yields a rectangular hyperbolic curve. At the O_2 tension of arterial blood (about 100 mm.Hg.) myoglobin is nearly 95% saturated. The resting capillary O_2 tension is about 40 mm.Hg. at the arterial end. In the active muscle, it may decrease to as low as 20 mm.Hg. Even at such low oxygen tensions myoglobin can be over 80% saturated with oxygen. The tissue oxygen tension during activity will become as low as 5 mm.Hg. and thus enough gradient exists for the myoglobin to supply oxygen to the tissues. It parts with about 12% of oxygen carried by it under these conditions.

In hemoglobin, four subunits are there in the molecule—alfa$_1$, alfa$_2$, beta$_1$ and beta$_2$ chains. The four subunits would display a 'cooperative' effect in transporting oxygen. Each alfa unit is more or less rigidly attached to a beta unit, but the attachment to the other beta unit is less rigid (*i.e.*, the attachment of alfa$_1$ to beta$_2$ and alfa$_2$ to beta$_1$. Even these less rigid attachments exhibit alterations between oxyhemoglobin and reduced hemoglobin. In the oxygenated state, these bonds become more relaxed (R state) and in the reduced state, these bonds are more taut (T state) because of breakdown of small salt bridges between the units in the R state, and their reformation in the T state. The $\alpha_2\beta_2$ dimer exhibits slight rotation (about 15°) in relation to the $\alpha_1\beta_1$ dimer during the conversion of the R form to the T form. In the R form, Fe^{++} atom would be exactly in the plane of the porphyrin ring. In the T form, it is 0.07 nm out of the plane. Also, a valine residue projects into the heme pocket of the beta chain in the T form and blocks the entry of oxygen. The valine residue gets retracted away from the pocket in the R form because of the slight rotation and loosening of the salt bridges.

Due to all these factors, the R form is having affinity for oxygen which is several hundred times that of the T form. Formation of the R form would be favoured when one or two oxygen atoms are able to occupy the heme pockets of the alfa units. Thus, the uptake of one or two oxygen atoms by the alfa units triggers the conversion of the T form to the R form and is able to increase the affinity of the hemoglobin molecule to oxygen several hundred fold.

The reverse set of reactions take place if the molecule loses oxygen. The salt bridges get reformed, the $\alpha_2\beta_2$ unit rotates back to the preoxygenated state, valine projects back into the heme pockets of the beta chains and the affinity to oxygen gets enormously reduced. Oxygen, therefore, rapidly gets dissociated from hemoglobin, and the reduced T form of hemoglobin gets formed.

Due to this cooperativity in taking up oxygen by the four subunits of hemoglobin, the O_2 dissociation curves for hemoglobin take an S-shape.

The main differences between the T form and the R form of hemoglobin are given in the table below :

T (Taut) form (reduced Hb)	*R (relaxed) form (oxyHb)*
1. $\alpha_1\beta_1$ and $\alpha_2\beta_2$ units are having their long axis close.	A rotation of 15% exists between the two.
2. Salt bridges are many.	Salt bridges are less in number.
3. Fe^{++} is 0.07 nm out of the plane of porphyrin ring.	Fe^{++} is in the plane of porphyrin ring.
4. Valine residue projects into heme pocket of beta chains.	Valine residue does not project. Heme pockets are free to take up O_2.
5. Affinity for O_2 has been quite low.	Affinity for O_2 has been high by several hundred fold.
6. Beta chain histidine residues get protonated (H^+ added).	Histidines of beta chains release protons ($2H^+$).
7. DPG may enter and could be retained by salt bridges in a central cavity formed between the four subunits.	DPG is unable to bind.

Factors Affecting the Dissociation of Oxyhemoglobin

1. Temperature

(a) A rise in temperature decreases hemoglobin saturation.

(b) At 25°C, hemoglobin is 88% saturated but at 37°C, it is only 56% saturated. Therefore, hemoglobin gives up oxygen more readily while passing from high to low oxygen more readily while passing from high to low oxygen tension (as from lungs to tissues) in warm-blooded animals than in cold-blooded animals.

2. Electrolytes

At low oxygen tensions oxyhemoglobin gives up oxygen more readily in the presence of electrolytes.

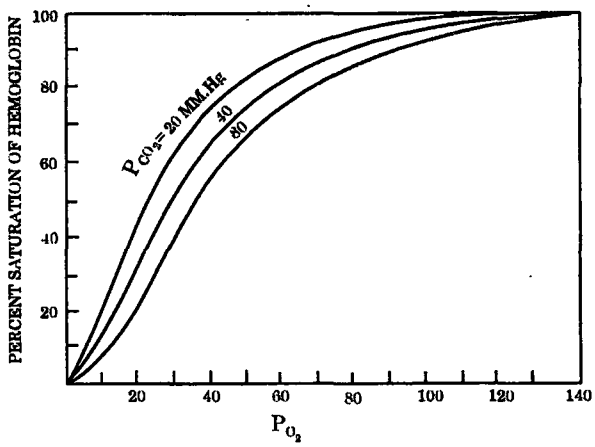


Fig. 31.1. The dissociation oxyhemoglobin.

3. Effect of CO_2

(a) The influence of CO_2 on the shape of the dissociation curve is actually the effect of carbonic acid formation with the lowering of the pH of the environment.

(b) The increase in acidity facilitates the dissociation of oxyhemoglobin.

(c) The ability of CO_2 to shift the slope of the oxyhemoglobin dissociation curve to the right is known as the *Bohr effect.* This effect is often described as causing a shift of the P-50 to the right. The P-50 is the partial pressure (mm.Hg.) at which hemoglobin is 50% saturated. The 2, 3-biphosphoglycerate, a compound formed during glycolysis in the red cells, also causes a significant shift of the P-50 to the right.

Carboxyhemoglobin

1. Hemoglobin undergoes combination with carbon monoxide more readily than with oxygen (210 times as fast) to form cherry-red carboxyhemoglobin.

2. This reduces the amount of hemoglobin to carry oxygen.

3. The carbon monoxide in the inspired air is 0.02%, headache and nausea occurs.

4. In case, the carbon monoxide concentration is only that of oxygen in the air (about 0.1% carbon monoxide), unconsciousness occurs in 1 hour and death in 4 hours.)

Role of Diphosphoglycerate : It acts as an allosteric regulator of hemoglobin activity. It occurs in the red cell in equimolar concentration to hemoglobin. The affinity of DPG to hemoglobin is much more (about two times as much) than for oxyhemoglobin. It binds allosterically hemoglobin molecules and brings about conformational changes similar to oxygen binding in tissue capillaries. The DPG levels get increased erythrocytes of people living at high attitudes.

CO_2 itself acts as an allosteric regulator by undergoing combination reversibly with the $—NH_2$ of the *n*-terminal amino acids to form carbamine hemoglobin. Hemoglobin is having a higher affinity for CO_2 than oxyhemoglobin. As the O_2 gets lost from the oxyhemoglobin, CO_2 is take up by the hemoglobin to form carbamino-hemoglobin.

Fetal Respiration : The fetus gets supply of oxygen from placental blood, the pO_2 of which is quite low. Still, the fetal hemoglobin (HbF) is able to draw oxygen from placental blood, because the O_2 dissociation curve of HbF is appreciably shifted to the left compared to adult hemoglobin. This is because of the low affinity of HbF to DPG. At a pO_2 of 30 mm Hg., while HbA is only 33% saturated with oxygen, HbF is somewhat 58% saturated.

Oxygen toxicity : Exposure to oxygen under high pressure gives rise to toxic symptoms and may prove fatal. Pure oxygen is an irritant to the lungs and gives rise to pulmonary edema. Oversaturation of hemoglobin with oxygen will prevent the transport of carbon dioxide, especially the isohydric phase. The –SH enzymes are also oxidized to the inactive -S-S- forms.

31.3 Transport of Carbon Dioxide in Blood

Carbon dioxide in solution forms carbonic acid which will lower down the pH to the acid side. But actually there occurs only slight lowering of pH by 0.01 to 0.03 in the conversion of arterial to venous blood, though large amounts (6.6 ml/100 ml of blood) of CO_2 are added to the venous blood.

As arterial blood enters the capillary bed in the tissues, it is having 48.2 vols% of CO_2 at a pCO_2 of 40 mm. This is separated by endothelial lining of capillary from the interstitial fluid which has a pCO_2 of around 50 mm. This gradient is sufficient to allow CO_2 to diffuse from the tissue fluid into the capillary blood. The CO_2 is first taken up as dissolved CO_2, but it is soon dealt with by other chemical mechanisms. The CO_2 content rises to 54.8 vols%/100 ml by the time it reaches the veins as venous blood.

In the lungs the reverse set of conditions prevail where the blood in the capillaries gets exposed to alveolar air with a pCO_2 of 40 mm. Carbon dioxide diffuses out into the alveoli by a reversal of the chemical processes through the stage of physical solution to part with the 6.6 ml gained for restoring the original CO_2 content and pCO_2 of arterial blood.

Mode of Transport of CO_2

1. Physical solution : The solubility of CO_2 in plasma and erythrocyte fluid at pCO_2 of 46 (venous blood) is 3.2 ml/100 ml and at pCO_2 of 40 (arterial blood), it is 2.7 ml/100 ml. This is responsible for transport of about 0.5 ml of CO_2 which is 7-8% of the 6.6 ml of additional CO_2 transported by venous blood.

2. Chemical transport : (i) Transport by plasma proteins : The CO_2 reacts with water to form carbonic acid, which in turn, dissociates to form the hydrogen and bicarbonate ions. The hydrogen ion is taken up by the protein buffers and the bicarbonate by the bases present in plasma.

$$H_2O + CO_2 \longleftrightarrow H_2CO_3 \longleftrightarrow H^+ + HCO_3^-$$
$$H^+ + \text{Protein} \longleftrightarrow \text{H. protein}$$
$$HCO_3^- + B^+ \longleftrightarrow B\,HCO_3$$

Other buffer systems such as the phosphate may also take up the hydrogen ion. This is able to account for 4% of the CO_2 transport (4% of 6.6 ml), *i.e.*, about 0.26.

Carbonic anhydrase : This enzyme is able to facilitate the formation of H_2CO_3 and its dissociation to form bicarbonate. It occurs in the erythrocytes and accounts for the formation of more than 90% of the HCO_3^- in the R.B.C.

(ii) Transport by hemoglobin of the erythrocytes : This occurs by two methods :

(a) Formation of carbaminohemoglobin : Some of the free amino group of hemoglobin readily combine with CO_2 in a reversible reaction to form carbaminohemoglobin.

$$Hb\ NH_2 + CO_2 \longleftrightarrow Hb\ NH_2\ COO^-$$

Reduced hemoglobin is having greater affinity for CO_2 than oxyhemoglobin. As the uptake of CO_2 and the release of O_2 go together, the conditions become highly favourable of carbaminohemoglobin formation. This accounts for 20% of the CO_2 transported (20% of 6.6 ml) *i.e.*, 1.3 ml.

(b) Isohydric transport of CO_2 by hemoglobin : Quantitatively this is the most important and accounts for about 70% of the CO_2 transported (70% of 6.6 ml, *i.e.* 4.6 ml). Further, the transport is managed without the slightest change in pH on this account (hence isohydric).

Haldane Effect : Binding of O_2 to hemoglobin displace CO_2 from it. This is called Haldane effect.

Both hemoglobin and oxyhemoglobin behave as weak acids. Between the two, oxyhemoglobin is stronger acid ($pK = 2.4 \times 10^{-7}$) and dissociates to yield more hydrogen ions than reduced hemoglobin ($pK = 6.6 \times 10^{-9}$). If the two hemoglobins are represented to indicate their acid nature as $HHbO_2$ and HHb, then the conversion of $HHbO_2$ to HHb which takes place during the passage of blood through the capillary bed of tissues will need addition of hydrogen ion to the weaker acid HHb formed. During this phase the reactions are taking place simultaneously due to intake of CO_2 from the tissues. The HHb will take up the H^+ ion and the HCO_3^- is taken up by the bases (K^+ mainly in the cell). This is called the isohydric transport of CO_2.

$$H_2O + CO_2 \longleftrightarrow H_2CO_3 \longleftrightarrow H^+ + HCO_3^-$$

31.4 Chloride Shift

1. CO_2 combines with water to form carbonic acid (H_2CO_3) mainly inside the red cell by the enzyme carbonic anhydrase present in the red cells.

2. The carbonic acid is then buffered by the intracellular buffers (phosphate and hemoglobin) combining with potassium.

3. Bicarbonate ion also returns to the plasma and exchanges with chloride which shifts into the cell when the tension of CO_2 increases in the blood.

4. When the CO_2 tension gets reduced, chloride leaves the cell and enters the plasma.

5. Under normal conditions the red cell is impermeable to sodium or potassium. But it is permeable to hydrogen, bicarbonate and chloride ions and intracellular sources of cation (potassium) are indirectly available to the plasma by chloride (anion) exchange. This allows the carriage of additional CO_2 (as sodium bicarbonate) by plasma.

6. The CO_2 entering the blood from the tissues enters the red cells where it forms carbonic acid by carbonic anhydrase. Some of the carbonic acid returns to the plasma. The remainder combines with hemoglobin buffer to form bicarbonate with then returns to the plasma in exchange of chloride. The chloride gets neutralized by potassium in the red cells.

7. All of these reactions are reversible. At the lung, when the blood becomes arterial, chloride shifts back into the plasma, thereby liberating intracellular potassium to buffer the oxyhemoglobin and in the plasma, neutralizing the sodium which gets liberated by the removal of CO_2 during respiration.

Significance of N_2 in Respiration : There exists no chemical mode of transport of nitrogen in blood. It occurs only in physical solution. At the pN_2 of 570 mm 0.9 ml of nitrogen is dissolved per 100 ml. This remains the same in arterial and venous blood and in tissues. When the pressure gets increased as in diving under the sea, more nitrogen gets dissolved.

If in such an individual, there occurs a sudden decompression, the dissolved nitrogen will get liberated as bubbles of nitrogen gas into the blood and tissues. In blood this may bring about embolism. In tissues, particularly nervous tissues, it will bring about severe pain due to pressure on the nerves. The individual has to be again kept in a compressed atmosphere and decompression should be carried out in a gradual manner to allow time for the nitrogen to be swept out through respiration.

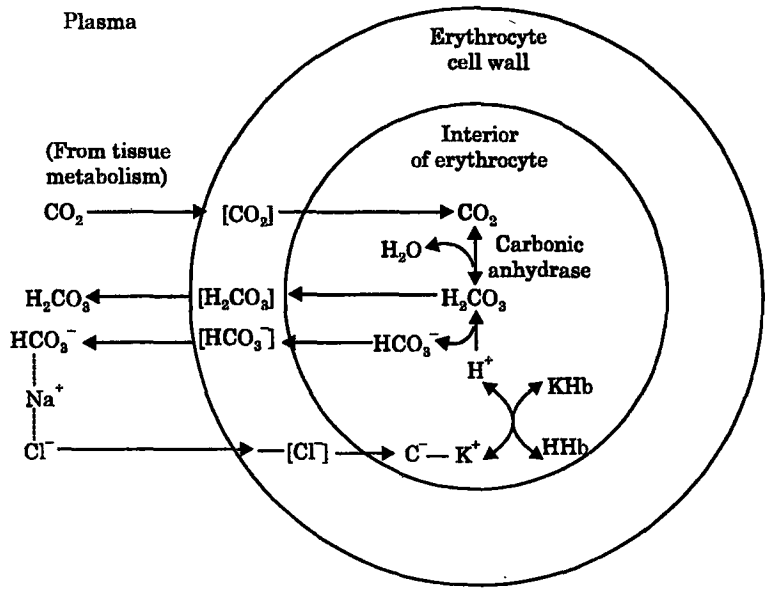


Fig. 31.2. Chloride shift.

31.5 Control of Respiration

Respiratory Centre : This is situated in the medulla oblongata and sends out impulses to the respiratory apparatus. Increase in pCO_2 and H^+ ion concentration is able to stimulate the respiratory centre directly. The rate and depth of respiration gets increased till the excess CO_2 is blown out, thereby allowing the pCO_2 and H^+ to return to normal. Decrease in pCO_2 and H^+ ion concentration depresses the centre and acts in a reverse way to conserve CO_2 and raise the H^+ ion concentration.

Chemoreceptors of the Carotid and Aortic Bodies : These are stimulated by a decrease in pO_2 and increase in pCO_2 and H^+ ion concentration of the arterial blood. The impulses are carried through nerves to the respiratory centre which is reflexly stimulated. The main stimulus for chemoreceptors has been pO_2 decrease.

There are certain perfluoro compounds like *perfluorodecalin* which can dissolve gases like O_2 and CO_2 proportional to their partial pressures. The dissociation curve is linear. They are also non-toxic, when used for short periods. Hence, in conditions which need partial or complete replacement of erythrocytes, emulsions of this compound are injected intravenously to temporarily serve the function of respiratory exchange of gases.

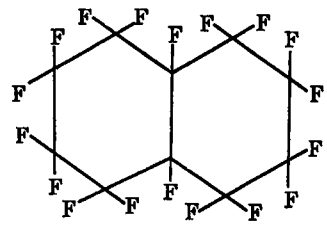


Fig. 31.3. Perfluorodecalin.

31.6 Buffer Systems of the Blood

1. It is known that venous blood carries more CO_2 than arterial blood. Therefore, the pH of venous blood is more acid than that of arterial blood by 0.01–0.03 units, *i.e.*, pH 7.40 and 7.43 respectively.

2. The blood buffers consist of the plasma proteins, hemoglobin, oxyhemoglobin, bicarbonates and inorganic phosphates.

3. When CO_2 enters the venous blood, the small decrease in pH is able to shift the ratio of acid to salt in all the buffer pairs. When the ratio is shifted to form more of the acid, cations, become available to form additional bicarbonates. In this respect, the plasma phosphates and bicarbonates would play a minor role.

4. The buffering action of the plasma proteins is important as they release sufficient cations for the carriage of about 10% of the total CO_2.

5. The phosphates in the red cells are known to carry 25% of the total CO_2.

6. The most important is buffering role of hemoglobin and oxyhemoglobin which carries 60% of the CO_2 of the whole blood.

Hemoglobin Buffers

At the *lungs* the formation of oxyhemoglobin from reduced hemoglobin releases hydrogen ions which undergo reaction with bicarbonate to form carbonic acid. The low CO_2 tension in the lungs shifts the equilibrium towards the production of CO_2 which is continually eliminated in the expired air.

$$H^+ + HCO_3 \longleftrightarrow H_2CO_3 \longleftrightarrow H_2O + CO_2$$

In the *tissues*, the oxygen tension gets educed and hence oxyhemoglobin dissociates delivering O_2 to the cells and reduced hemoglobin is formed. The CO_2 produced by metabolism enters the blood, where it gets hydrated to form H_2CO_3 which ionizes to form H^+ and HCO_3^-. Reduced hemoglobin acting as an anion accepts the H^+ ions forming acid-reduced hemoglobin (HHb). Very little change in pH takes place because the newly arrived H^+ ions are buffered by formation of very weak acid.

When the blood returns to the lungs, these H^+ ions are released because of the formation of a stronger

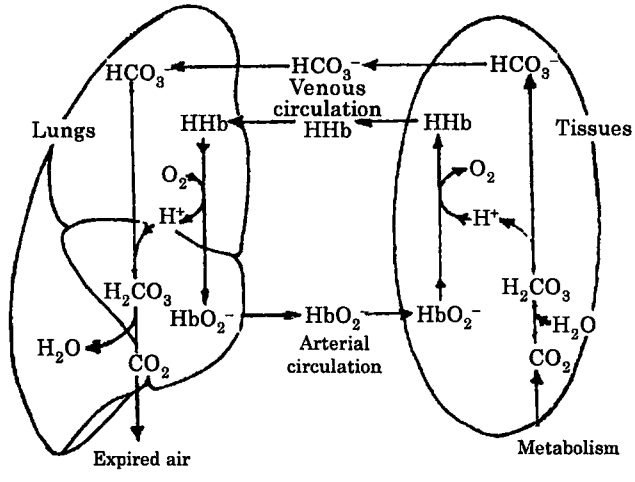


Fig. 31.4. The buffering action of hemoglobin.

acid (oxyhemoglobin) and the newly released H^+ ion gets promptly neutralized by HCO_3^-. This reaction has been found to be necessary for the liberation of CO_2 in the lungs.

Acid-Base Balance

Respiratory acidosis—This conditions arises if there occurs the accumulation of H_2CO_3 in the blood under certain circumstances.

Respiratory alkalosis— This condition arises if the rate of elimination of CO_2 is excessive following the reduction of H_2CO_3 in the blood.

Metabolic acidosis— This happens in the deficit of bicarbonate without any change in H_2CO_3.

Metabolic alkalosis— This occurs in the excess of bicarbonate.

Causes of Disturbances in Acid-Base Balance

A. Metabolic Acidosis

1. It happens in uncontrolled diabetes with ketosis.
2. It happens in some cases of vomiting when the fluids lost are not acid.
3. It also happens in renal disease, poisoning by an acid salt, excessive loss of intestinal fluids (as in diarrhea or colitis) and excessive losses of electrolytes.
4. Increased respiration is an important sign of uncompensated acidosis.

B. Respiratory Acidosis

1. This takes place in any disease which impaires respiration such as pneumonia, congestive failure, asthma.
2. It also occurs in the depression of the respiratory center (as by morphine poisoning).
3. A poorly function respiratory also gives rise to this condition.

C. Metabolic Alkalosis

1. Causes

(a) Due to the ingestion of large quantities of alkali in the treatment of peptic ulcer.

(b) In the high intestinal obstruction (as in pyloric stenosis).

(c) After prolonged vomiting or after the excessive removal of gastric secretions containing hydrochloric acid.

(d) In Cushing's disease and during corticotropin or cortisone administration.

2. Symptoms

(a) Tetany is caused because of the decrease in ionized serum calcium.

(b) The respirations are slow and shallow.

(c) The urine becomes alkaline.

(d) Elevated blood bicarbonate.

(e) Deficiency of potassium.

D. Respiratory Alkalosis

1. This is caused by hyperventilation.
2. It may occur in patients in hepatic coma.

Role of the Kidney in Acid-Base Balance

1. The non-volatile acids including lactic and pyruvic acids, hydrochloric acid, phosphoric acid and sulphuric acid get produced by metabolic processes. About 50-150 mEq of the

inorganic acids are eliminated by the kidneys in 24 hours. These acids are partially buffered with cations, largely sodium. In the distal tubules of the kidney some of the cations get reabsorbed and the pH of the urine falls.

2. Kidney can buffer acids and conserve fixed base in the production of ammonia from amino acids. The ammonia is used to neutralize acids when formed in excess.
3. In kidney disease, tubular reabsorption of sodium in exchange for hydrogen is poor and there occurs excessive retention of phosphates and sulphates. As a result acidosis takes place.

31.7 Hypoxia

This term means oxygen deficiency in any condition of insufficiency of tissue oxidation process. Four types of hypoxia are known :

1. Hypoxic hypoxia
2. Anemic hypoxia
3. Stagnant hypoxia
4. Histotoxic hypoxia.

1. Hypoxic Hypoxia

It is characterized by normal oxygen capacity but adminished oxygen tension in the arterial blood thereby resulting of varying degree of hemoglobin unsaturation.

(a) High altitude : Hypoxia is caused by the diminished oxygen tension in the atmospheric air and consequently in the alveolar air and blood stream. This gives rise to respiratory alkalosis.

(b) Rapid, shallow respiration : Shallow breathing is due to insufficient oxygenation of blood. This happens in case of diseases.

(c) Congenital heart disease : In the congenital cardiac defect a portion of the blood may flow directly from the right to the left side of the heart without passing through the lungs. Because of this, the mixture of aerated and non-aerated blood in the systemic circulation brings about hypoxic hypoxia.

2. Anemic Hypoxia

It is characterized by a diminution in the oxygen capacity of arterial blood because of the decrease in the amount of functioning hemoglobin.

3. Stagnant Hypoxia

It is due to circulatory insufficiency, the rate of blood flow through the tissues being retarded, with resulting increase in the percentage volume of oxygen removed from the blood in its passage through the capillaries.

4. Histotoxic Hypoxia

In this condition the oxygen supply becomes normal in every respect but the degree of oxygen utilization by the tissues gets diminished. The reason for this is that the tissue cells are poisoned in such a way that they cannot use oxygen properly.

32 Excretion

32.1 Introduction

Living cell is in a condition of constant change; chemical reactions are continually taking place within it. Reactions involving the release of energy by respiration go on in everything which is alive. Other reactions concern the alteration of one kind of material into another. Even the apparently permanent structures of the body like muscles, blood or skin are, in fact, changing from day-to-day. The chemical units of living protoplasm are constantly being renewed. New molecules are being added, and defective molecules or entire cells are being digested away. All these reactions give rise to end-products, some of which are poisonous above a certain concentration and which could affect the normal functioning of the body if they were allowed to accumulate.

Excretion is the process by which such toxic products are removed from the body before they can accumulate to harmful levels.

The main *excretory products* in animals are carbon dioxide and water produced as end-products of respiration and nitrogenous compounds from the breakdown of amino acids. Part of this nitrogenous material arises from the deamination in the liver of amino acids absorbed after a meal containing more protein than the body needs, and part from the breakdown of protein in the renewal and repair of the protoplasm of the body. The products of amino acid breakdown are ammonia and other nitrogen-containing compounds which could, if allowed to accumulate in the body, cause death in a matter of days or weeks. They are converted in the liver to *urea* and *uric acid,* which are less poisonous.

In man and other mammals the excretory products of the body's cells pass into the tissue fluid surrounding them and thence into the blood, which carries them to the *excretory organs,* the lungs, the liver and the kidneys. The lungs excrete carbon dioxide in exhaled air, the liver

excretes bile pigments derived from haemoglobin decomposition into the alimentary canal, where they are incorporated eventually into the faeces, and the kidneys remove nitrogenous material and excess mineral salts and water from the blood and eliminate them in the urine.

The kidney is an organ in very close *contact* with the blood. The kidney is a *homeostatic organ* maintaining the concentration of water by *osmoregulation,* removing urea by *excretion,* and regulating the concentration of the blood mineral ions and also its pH, acidity or alkalinity.

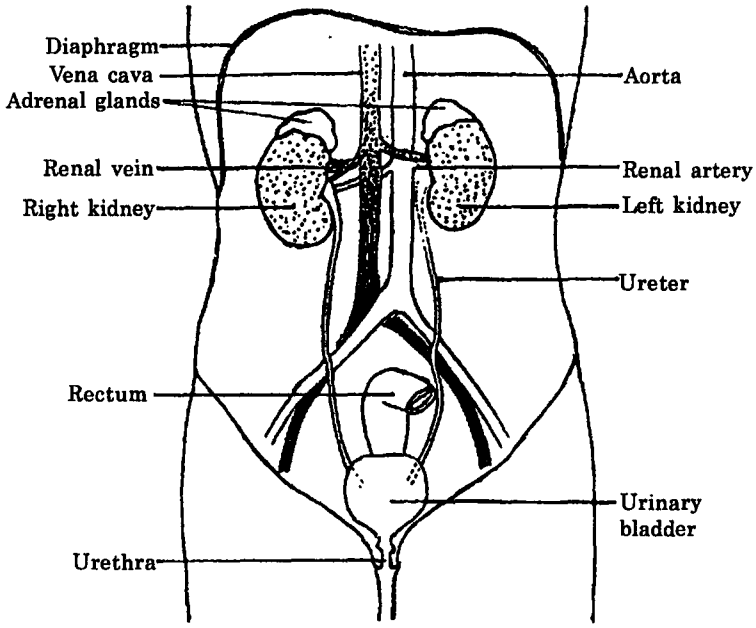


Fig. 32.1. Position of kidneys in the body.

32.2 Structure of Kidneys

The two kidneys are fairly solid structures attached to the back of the abdominal cavity (Fig. 32.1). They are oval in shape, with an indentation on their innermost sides, and red-brown in colour. Each kidney weighs about 250g, and is enclosed in a transparent membrane. The kidneys receive oxygenated blood through a branch of the aorta, the *renal artery* (*renal* means 'of the kidney'), and deoxygenated blood is carried away from them in the r*enal vein* to the inferior vena cava. Close to these blood vessels are the paired adrenal glands. A tube, the *ureter,* runs from each kidney to the base of the *bladder* in the lower abdomen.

Tubular *ureters* convey urine by *peristalsis* from each kidney into the *urinary bladder,* which is emptied by way of the *urethra* during *micturition.* The urethra is controlled by a ring-like *sphincter* muscle.

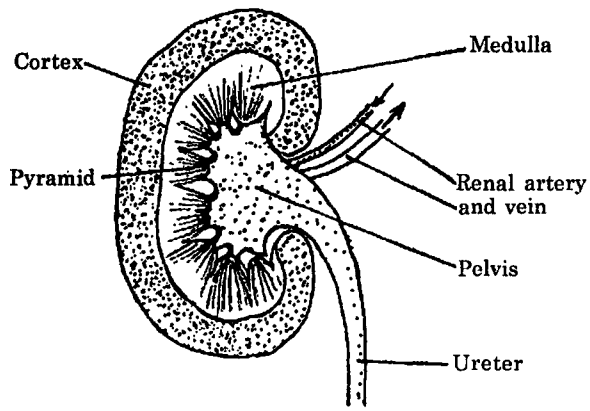


Fig. 32.2. Section through kidney to show regions.

A section through a kidney (Fig. 32.2) shows a darker, outer region, the *cortex,*

an inner zone which is lighter in colour, the *medulla,* and a hollow space called the *pelvis* from which the ureter leads. Cones or *pyramids* of kidney tissue project into pelvis.

The kidney tissue consists of many minute tubes, *renal tubules,* and capillary blood vessels, all held together by connective tissue. The renal artery divides up into a great many arterioles, mostly in the cortex (Fig. 32.3). Each arteriole leads to a *glomerulus,* which is a little knot of capillary blood vessels, repeatedly divided and coiled. Each glomerulus is almost entirely

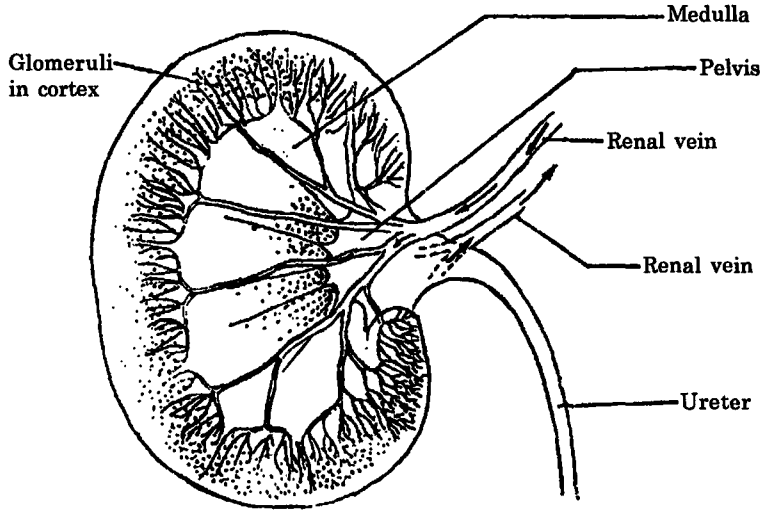


Fig. 32.3. Section through kidney to show distribution of glomeruli.

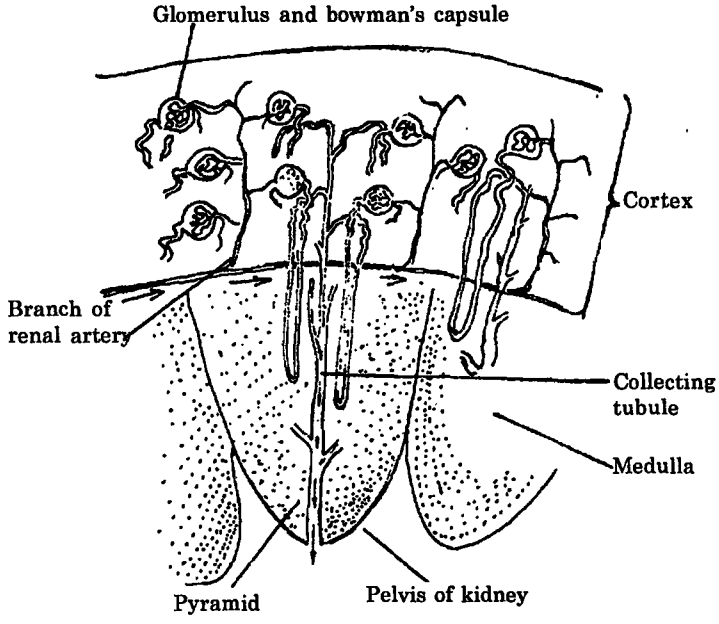


Fig. 32.4. Section through cortex and medulla.

surrounded by a cup-shaped organ, called a *Bowman's capsule,* about 0.1 to 0.2 mm in diameter, which leads to a renal tubule. This tubule, after a series of coils and loops, joins up with other tubules to form a *collecting tubule* which passes through the medulla to open into the pelvis at

the apex of a pyramid. The capillaries from the glomeruli and the renal tubules unite to form the renal vein (Fig. 32.4).

Nephron

The *nephron* is the basic unit of the kidney, there being over a million in each kidney. The renal artery supplies an *afferent arteriole* which connects with a small bunch of blood capillaries called a *glomerulus* which projects inside a *Bowman's capsule.* Each Bowman's capsule is the blind end of the kidney *tubule* which is made up of a *first* convoluted tubule, *descending* and *ascending* tubules with a loop of Henle in between, and a *second* convoluted tubule leading to a *collecting duct.* An *efferent arteriole,* which has a narrower bore than the *afferent arteriole,* removes blood from the glomerulus to supply a network of blood vessels to the tubule before returning the blood into the *renal vein.*

32.3 Mechanism of Kidney Excretion

The narrow and tortuous capillaries of the glomerulus offer considerable resistance to the flow of blood, so that a high pressure is set up. This is sufficient to overcome the osmotic pressure of the blood plasma so that fluid filters out through the capillary walls in the glomerulus and collects in the Bowmen's capsules.

The filtered fluid, *glomerular filtrate,* has a composition similar to that of blood serum, containing glucose and amino acids, salts and nitrogenous waste products; fibrinogen and other plasma proteins remain in the blood. The filtration process is continuous; in man, 180 litres of the filtrate, carrying 145 g glucose and 1100 g sodium chloride, pass each day into the Bowman's capsules. As the filtrate passes down the renal tubule, all the glucose and amino acids, some of the salts and much of the water are absorbed back into the network of capillaries that surround the tubule (Fig. 32.5). This selective reabsorption prevents the loss of useful substances from the body. The remaining liquid, now called *urine,* contains only the waste products such as urea and related substances, hormones which have been inactivated in the liver, excess salt and water.

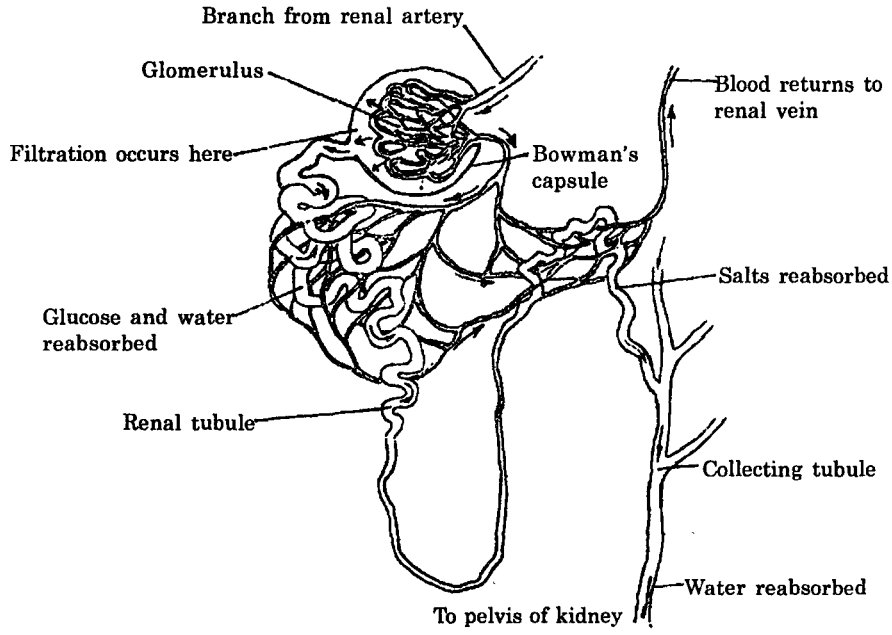


Fig. 32.5. Diagram of glomerulus and Bowman's capsule.

The urine passes down the collecting tubule, where more of the water is reabsorbed. The

amount of water removed from the urine is controlled very precisely by hormone action in which a way that the concentration of the blood is regulated. If the blood is dilute, as it is when a great deal of liquid has been drunk, comparatively little water is reabsorbed, the urine is therefore dilute and the excess water is eliminated. If, on the other hand, the blood is concentrated, which may happen after loss of water by profuse sweating more water is absorbed in the collecting tubule and less water is lost in the urine. From the collecting tubule the urine enters the pelvis of the kidney, whence it passes down the ureter to the bladder as a result of waves of contraction moving along the ureter walls.

The selective reabsorption from the glomerular filtrate is performed by the cells of the walls of the kidney tubules. It often proceeds against a diffusion gradient by mechanisms which are not fully understood but which certainly need a supply of energy, obtained by respiration within the cells. The blood leaving the kidneys in the renal vein therefore contains less oxygen and glucose and more carbon dioxide, as a result of tissue respiration, and less water, salts and nitrogenous waste, as a result of excretion, than the blood in the renal artery.

Table 32.1 lists the main nitrogenous compounds removed from the blood by the kidneys.

Table 32.1

APPROXIMATE PERCENTAGE OF NITROGENOUS COMPOUNDS IN BLOOD PLASMA AND URINE

	Blood plasma	*Urine*
Proteins	7-9	0
Urea	0.03	2
Uric acid	0.004	0.06
Ammonium compounds	0.0001	0.04
Water	90-93	95]

32.4 Bladder

The bladder is an extensible sac with elastic and muscular tissue in the walls. The accumulating urine entering the bladder from the two ureters expands its elastic walls to a volume of 400 cm^3 or more. At intervals the muscles in the bladder walls contract, while at the same time the sphincter muscle closing the bladder outlet relaxes, so that urine is expelled from the body through a duct called *urethra;* the action is assisted by pressure from the muscle of the abdominal wall.

In young babies, the relaxation of the sphincter muscle is a reflex action brought about by nerve impulses triggered off by the stretching of the bladder walls. After the age of two years or so, the relaxation of the muscle can be controlled voluntarily.

32.5 Water Balance and Osmoregulation

Water is lost from the body in urine, faeces, sweat and exhaled breath. It is gained in food and drink. These losses and gains produce corresponding changes in the blood.

Changes in the concentration of the blood are detected by the *hypothalamus,* the part of the brain. If the blood passing through the brain is too concentrated, the hypothalamus stimulates the *pituitary gland,* which lies just beneath it to secrete a hormone called *antidiuretic hormone* (ADH). When this hormone is carried in the blood to the kidneys it stimulates the reabsorption of water from the glomerular filtrate by the cells in the walls of the kidney tubules. Thus the urine becomes more concentrated and the further loss of water from the blood is reduced. If, on

the other hand, the blood passing though the hypothalamus is too dilute, production of ADH by the pituitary gland is suppressed and less water is absorbed from the glomerular filtrate.

The intake of water by the body is controlled by the sensation of thirst, which stimulates the individual to take in fluid. The mechanism producing feelings of thirst is not well understood, but it undoubtedly serves to regulate the intake of water and so to maintain the concentration of the blood within certain limits.

The kidneys have an important part in the homeostasis of the body in the three ways in which they control the composition of the blood :

(*a*) they remove urea and other harmful substances;

(*b*) they remove excess water;

(*c*) they remove excess mineral salts.

These activities are both excretory, in that they remove the unwanted products of metabolism, and osmoregulatory, in that they keep the osmotic potential of the blood more or less constant.

32.6 Homeostatic Functions of the Kidney

The kidney has *three* main homeostatic functions, in maintaining the pH, mineral ion and water composition of the blood fluid.

(i) pH regulation : The kidney tubule exchanges certain hydrogen or hydroxyl ions, to maintain the blood plasma fluid at a norm of pH 7.4. The exchanged ions in the urine cause it to be either acid at pH 5 or alkaline at pH 8. Urine pH can be shown by dipping a BDH Universal Indicator paper into a urine sample and matching the colour formed with the pH colour chart.

(ii) Mineral ion regulation : Sodium ions are controlled in a complex homeostatic process involving the adrenal gland hormone *aldosterone* which increases sodium ion absorption in the convoluted tubule.

(iii) Water balance control : The adult human body contains 45 litres of water which forms 65 to 70% of the body weight. This water is divided as follows :

(*i*) Inside the cells :	66%	30 litres;
(*ii*) Tissue fluid :	26%	12 litres;
(*iii*) Blood plasma :	6%	3 litres.

The water concentration of the *tissue fluid* is maintained within the range 97-99% (or mean 98%) by making the daily water *intake equal* the daily water loss.

Table 32.2 shows the daily water loss and gain, in a healthy person, at rest in a temperate climate. The *metabolic* water is the product of respiration of lipids, proteins and carbohydrates, 100 g of each producing 100 cm^3, 40 cm^3s and 60 cm^3 of water respectively.

Homeostatic mechanisms operate when insufficient or excessive water intake occurs.

(1) Insufficient water intake

(*i*) A shortage of water causes the water concentration to fall *below* the norm of 98%; this in turn causes the blood *osmotic pressure* to rise.

(*ii*) Sensory receptors in the brain (*hypothalamus*) detect the change in *osmotic pressure* and signal the *pituitary gland* by negative feedback.

(*iii*) The pituitary gland sets off the corrective mechanism by secreting the *antidiutetic hormone* (ADH) or *vasopressin;* this causes the kidney tubule to absorb *more* water. The urine becomes *scanty* and *concentrated.* Thirst develops and water is taken into the blood from the intestine diluting the blood and returning the water composition to its norm of 92%.

Table 32.2

DAILY WATER LOSS AND GAIN IN MAN (IN GOOD HEALTH, AT REST, IN TEMPERATE CLIMATE)

Loss (cm^3)			*Gain* (cm^3)		
1. By skin, sweat	500	(20%)	1 In drink	1500	(60%)
2. Lungs, exhalation	400	(16%)	2 In food	700	(28%)
3. In faeces	100	(4%)	3 Metabolic water,		
4. In urine	1500	(60%)	a product of respiration*	300	(12%)
	Total = 2500 cm^3			Total = 2500 cm^3	

*Glucose + oxygen = carbon dioxide + *metabolic water.*

(2) Excessive water intake

(*i*) Excessive water intake *increases* the tissue fluid water concentration above the norm of 98%; this causes the blood osmotic pressure to *fall.*

(*ii*) Sensory receptors in the brain detect the osmotic pressure change and signal the pituitary gland by negative feedback.

(*iii*) The gland sets off a corrective mechanism which *stops* the gland secreting ADH or vasopressin. No more water is absorbed in the tubules, and the urine becomes copious and dilute. The tissue fluid concentration decreases and returns to the norm of 98% and blood osmotic pressure rises to its norm.

Water diabetes is due to defective water control through *positive feed back* and lack of ADH or vasopressin secretion. This causes production of large volumes of dilute urine and of thirst, leading to body *dehydration.*

Oedema or *dropsy* is the accumulation of water in body tissues.

32.7 Osmoregulation in Plants

There are no specific osmoregulation organs in higher plants. Control of water intake and loss is by means of those internal and external factors which affect the rate of *transpiration.*

Plants share with animals the problems of *obtaining* water, and in *disposing* of the surplus. Certain plants develop methods of water conservation.

Xerophytes are plants in dry habitats, deserts, which are able to withstand prolonged periods of water shortage. *Succulent* plants, such as the cacti, have water stored in large parenchyma tissue.

Other plants have leaf modifications to reduce water loss, such as needleshaped leaves, sunken stomata and thick waxy cuticles as in the pine. The stand-dune marram grass has rolled leaves with stomata on the inner surface.

32.8 How Does Kidney Functions ?

The kidney is a device for actively reabsorbing, from a protein-free filtrate, ions whose conservation is important to the body—Na^+s and other cations, Cl^- and to some extent HPO_4^{2-}, and also glucose and amino acids. Secondly, it reabsorbs almost all the water presented to it, a vital function for a terrestrial animal. Thirdly, it secretes a number of substances unwanted by the body, notably H^+ and NH_4^+, and in doing so helps regulate the acid-base balance of the body fluids.

These functions are highly energy-intensive; at rest the kidneys consume about 7% of the total O_2 used by the body. The only primary coupling mechanism between metabolic energy and

reabsorptive processes so far discovered is the Na^+/K^+ pump. This is very concentrated in the kidney, especially in the cortex (ascending limb of loop of Henle). The pump appears to be located entirely on the basement membrane; it is absent from the luminal side of the cells (apical membrane). This is important as it allows Na^+ entry from the lumen to be coupled in a number of ways (Fig. 32.6).

There is a discrepancy between estimates of the activity of the Na^+/K^+ pump and the turnover of ATP, calculated from the QO_2. Present estimates are that about 50% of the Na^+ entry is linked directly to ATP hydrolysis; perhaps 25% could be exchanged for H^+ (Fig. 32.6), but the remaining 25% is unaccounted for, energetically speaking. There is good evidence that in the thick ascending loop of Henle, the apical membrane contains an active Cl^- pump, which brings Na^+ into the cells with it. In view of the absence of a Ce^- reservoir in the body, an active reabsorption of Cl^- is to be expected. However, even at this point the overall pumping of NaCl probably depends on the extrusion of Na^+ through the basement membrane.

It is known that the formation of H^+ ions in the proximal convoluted tubules and elsewhere in the kidney depends on carbonic anhydrase activity (Fig. 32.6), since inhibitors of this enzyme prevent acidification of the urine and lead to the appearance in it of HCO_3^- ions. However, nothing is known of the mechanism of H^+ transport across the apical membrane, or whether a simple Na^+/H^+ exchange would be energetically favourable.

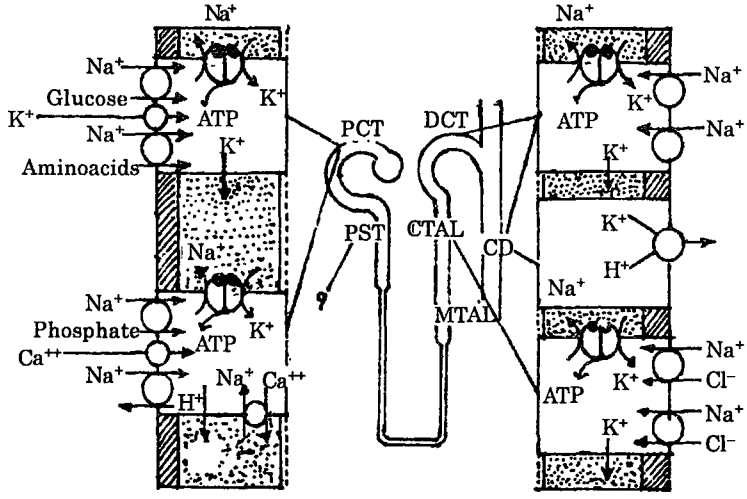


Fig. 32.6. Reabsorption and exchange mechanisms in the kidney, and their probably relation to the Na^+/K^+ pump. PCT = proximal convoluted tubule; PST = proximal straight tubule; MTAL, CTAL = medullary and cortical thick ascending limb; DCT = distal collecting tubule; CD = collecting ducts. The substance reabsorbed in the thick ascending limb is shown as NaCl. However, it is agreed that the primary process here [see Fig 32.7 (*a*)] is a chloride pump, although it is powered by a Na^+/K^+ ATPase, and Na^+ secondarily enters the tubule cells at this point.

In the proximal tubules, glucose and amino acids are actively absorbed by co-transport with Na^+, as in the small intestine. Possibly amino acids are also absorbed by the α-glutamyl transferase mechanism described earlier. Phosphate and Ca^{2+} are also actively reabsorbed. Most of the K^+ in the filtrate also appears to be reabsorbed in these tubules, so that the K^+ in the filtrate also appears to be reabsorbed in these tubules, so that the K^+ present in urine largely arrives by secretion (in competition with H^+) in the distal tubules and collecting ducts.

Countercurrent Mechanism

About 80% of the solids in the glomerular filtrate are absorbed in the proximal tubule, and about 80% of the H_2O follows the solutes, leaving a fluid roughly isotonic with plasma. Urine is however hypertonic. The final concentration of the tubular fluid is carried out by a countercurrent mechanism, illustrated in Fig. 32.7. The mechanism depends on the establishment of a solute concentration gradient, running from cortex to medulla, maintained by differences in permeability of the tubules at various points.

The active part in this process is played by the ascending tubule, particularly the thick-walled part, and by the collecting tubule. The whole of the ascending tubule is almost impermeable to H_2O, and the upper (thick-walled) part possesses the Cl^- and Na^+ pump referred to earlier (see Fig. 32.6). This has the effect of forming in the distal tubule a rather dilute urine, in which the major solute is urea.

By contrast, the collecting tubule is permeable to H_2O, and, progressively towards the collecting duct, to urea also (but not to NaCl). The effect is to produce an increasingly hypertonic urine, in which the urea concentration rises eventually to about 750 mM. The matrix surrounding the tubules is hypertonic; at the upper (cortical) end because of NaCl (concentration 4-600 millionmoles), and in the lower (medullary) end because of a gradient of urea rising to 500 milliosmoles, superimposed on the hypertonic NaCl solution. The total osmolarity at the bottom of the loop of Henle is thus about 1000 mOsm.

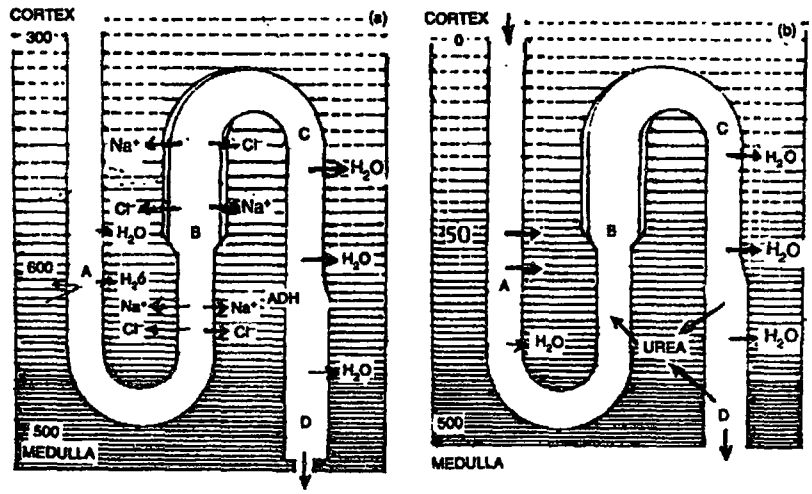


Fig. 32.7.The countercurrent mechanism for concentration of urine.

(*a*) Effect of the chloride and sodium pumps on the external osmolarity around the loop of Henle and the collecting ducts. Note that the descending limb of the loop (A) and the collecting tubule (C-D) are permeable only to H_2O, which moves out of the lumen to the higher osmolarity in the surrounding tissue. At the beginning of the ascending loop, the filtrate has became concentrated, so that Na^+ and Cl^- can move outward by diffusion, but in the thick ascending limb (B) the remaining Na^+ and Cl^- ions are pumped out against a concentration gradient. The figures in the left-hand margin refer to the osmolarity with respect to NaCl.

(*b*) Effect of urea permeability on the external osmolarity around the loop of Henle and the collecting ducts. It is mainly the collecting ducts (D) that are permeable to urea, which flows out from the urine, in which it has become concentrated. The extratubular fluid thus becomes very hyperosmolar in the inner cortex and medulla of the kidney, which assists water loss from the tubular fluid as it flows from A to B. The figures in the left-hand margin refer to the osmolarity with respect to urea.

We may now return to the thin descending loop of Henle, in which the fluid is originally isotonic, but loses water as it flows down into the cortex, so that at the bottom of the loop its concentration is also 1000 mOsm. As it enters the ascending limb, it loses some NaCl and gains some urea by diffusion, but as already mentioned, does not gain H_2O.

The channels for water and for urea in the distal tubules and collecting ducts are controlled by vasopressin, which opens them so that water moves out of the ducts. Vasopressin is known to increase adenyl cyclase activity in the membrane and hence the concentration of cAMP. There is increasing evidence that cAMP activates the phosphorylation of one or more membrane proteins, which presumably alters the configuration of the membrane, but the precise details of the way in which permeability is changed are not known at the present time.

32.9 Control of Acid-Base Balance

Acidosis

The formation of acid, *i.e.*, proton-donating, groups is more common in the body than is the formation of bases, *i.e.*, proton acceptors. The body's defences against acidosis, by the bicarbonate/CO_2 system, and by other mechanisms, are more efficient than the defences against alkalosis. A mild degree of acidosis is almost a normal condition; the oxidation of the —SH of cysteine to sulphate, together with the hydrolysis of phospho-diesters, *e.g.*, nucleic acids, to inorganic phosphate, are a continuous source of non-volatile acid. On a largely vegetarian diet the oxidation of salts of acids such as citrate and malate, effectively to $KHCO_3$, may on the other hand induce a mild alkalosis.

These disequilibria of dietary origin are not serious enough to cause concern. There are processes which release acid into the blood (acidaemia) in sufficient amounts to activate the physiological defence mechanisms; they can be either acute or chronic. The most common are lactic acidosis, usually from intense exercise, and keto-acidosis which is usually only serious if it arises from poorly-controlled diabetes. Other chronic acidosis can arise from inborn errors of metabolism, such as phenylketonuria or maple syrup urine disease, but in these the acidosis is usually less important than the other consequences.

(a) Respiratory response : When acid metabolites enter the blood the pH falls, and the ratio $[HCO_3]/[CO_2 + H_2CO_3]$ also falls. Stated in another way, the reactions

$$HA \rightleftharpoons H^+ + A^-$$
$$H^+ + HCO_3^- \rightleftharpoons H_2CO_3$$

occurs. Their equilibria are very far to the right. H_2CO_3 dissociates almost completely into H_2O and CO_2 and pCO_2 increases. This increases the amount of CO_2 blown off in the lungs. This brings the ratio $[HCO_3^-]/[CO_2 + H_2CO_3]$, which is equivalent to the ratio $[HCO_3^-]/qpCO_2$ back to normal, and the pH returns to normal.

The acidosis has been *compensated* by a respiratory response, but the composition of the extracellular fluids is not normal because the absolute concentration of HCO_3^- has been reduced, and the anion A^- of the acid metabolite is still present. If the acidosis is only temporary, the metabolite may be metabolized (as after exercise) or else excreted. The acid-base status then slowly returns to normal with less CO_2 being blown off in the lungs, and the $[HCO_3^-]$ in extracellular fluids rises to its normal level.

This description has concentrated on the response of the bicarbonate/CO_2 system. There are other buffers in blood and extracellular fluid which increase the resistance of the body to changes in $[H^+]$. The most important of these is hemoglobin. Each molecule contains 36 histidyl residues, many of which have *p*K's close to 7.4; only 4 of them are involved in the change of acidity of Hb

on oxygenation. The power of the others is quite independent of the state of oxygenation of the red cells. Plasma proteins also contain histidyl residues, but they are not quantitatively so important as those of hemoglobin.

From the Henderson-Hasselbalch equation it is possible to determine the acid-base status of an individual by measuring any two of the variables pH, total CO_2 and pCO_2. In interpreting the results, for example by the Siggaard Andersen nomogram, it will be observed that the hemoglobin content of the blood is taken into account. This is in order to allow for the extra buffering power of the histidyl residues, as described in the previous paragraph.

Apart from the pH itself (values outside the range 7.0-7.6 are barely compatible with life), the amount of Bronsted base (proton acceptors) still remaining in the blood and extracellular fluid is the most important quantity, because this determines the remaining capacity of the respiratory acid-base system, including the blood proteins to respond to further acidotic episodes, It is usual to specify an HCO_3^- concentration of 24 mEq/litre at pH 7.4, or a pCO_2 of 40 mmHg, as normal. A concentration of HCO_3^- below this level, when corrected to pH 7.4, is characterized as a *base deficit*. The base deficit read from the nomogram will be somewhat larger than the HCO_3^- below this level, when corrected to pH 7.4, is characterized as *base deficit*. The base deficit read from the nomogram will be somewhat larger than the HCO_3^- below deficit read from the nomogram will be somewhat larger than the HCO_3^- deficit, because of the hemoglobin (and plasma protein) supplementation.

This explanation of response to acidosis has been written in terms of an acid metabolite arising in tissues and accumulating in blood. There can also be a *respiratory aciosis,* in which CO_2 is not removed at the normal rate from the lungs. This may be due to acute respiratory failure, drug-induced respiratory paralysis, or lung congestion. In these situations pCO_2 will be high with pH low, but $[HCO_3^-]$ will be normal or even slightly high.

(b) Renal response : It is clear that in respiratory acidosis the acid-base status of the blood cannot be returned to normal by a physiological response of lung function. If the kidneys are functioning normally, it is they which compensate for the disturbance. The kidneys also play a part in compensating acidosis of non-pulmonary origin, unless the latter are very short-lived. A brief outline of the mechanism of kidney function is included at the end of this chapter. Here the chemical events will be summarized, with the kidneys treated as a 'black box.'

The response to respiratory acidosis provides the simplest picture. From the Henderson-Hasselbalch equation, one may infer that the pH will be restored to normal if the concentration of HCO_3^- increases, so that the ratio $[HCO_3^-]/[CO_2 + H_2CO_3]$ returns to normal. In ordinary circumstances, HCO_3^- is completely absorbed from the glomerular filtrate (for a mechanism see Fig. 32.8). Any increase in plasma $[HCO_3^-]$ therefore depends on two factors. The first is exchange of H^+ for Na^+, so that in effect the tubule cells are manufacturing H_2CO_3 from endogenous CO_2 (see Fig. 32.8), but are secreting $NaHCO_3$ into the plasma. The second factor (usually neglected) is the constancy of the osmotic pressure of plasma and ECF; this demands that an increase of $[HCO_3^-]$ must be balanced by a decrease of $[Cl^-]$. There must therefore be an increase in the net excretion of Cl^- into urine.

Metabolic Acidosis

As we have seen, the respiratory response to a mild metabolic acidosis may leave the blood pH completely normal, but there will be a base deficit, a lowered pCO_2, and an excess of the corresponding anion in the plasma. If this anion is lactate, it will be removed from plasma, chiefly by liver. Other anions must be excreted, but excretion is not normally a problem—the ion is

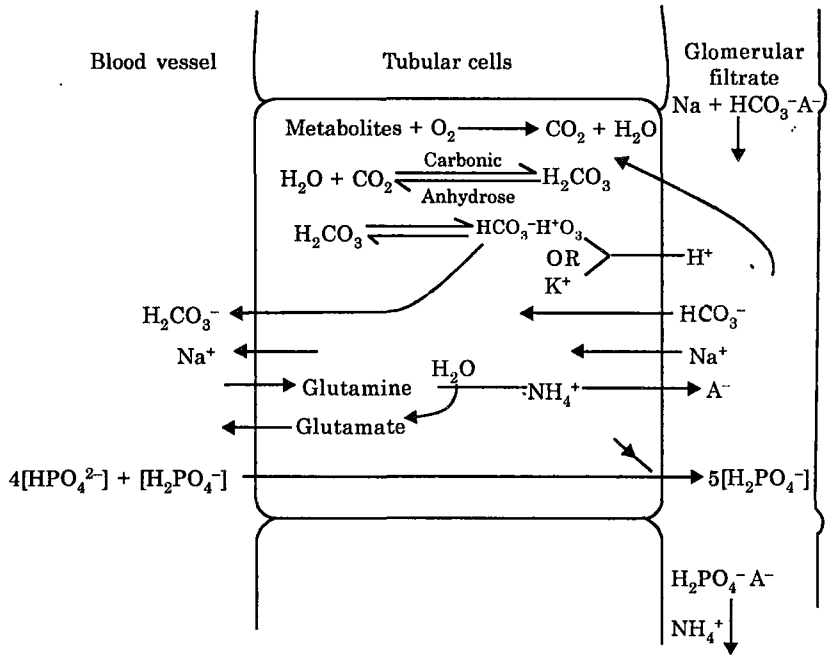


Fig. 32.8. The acidification of urine and ammonium ion excretion by the kidney. The diagram shows reabsorption of HCO_3^- both by diffusion into the tubular cells and by conversion to H_2CO_3 ($H_2O + CO_2$). NH_4^+ is shown exchanging with Na^+; the latter also exchanges with H^+ or K^+. The diagram also shows buffering of H^+ by excretion of HPO_4^{-2} and its conversion to $H_2PO_4^-$. The permanent anion A is not reabsorbed, and leaves the kidney largely neutralized by NH_4^+.

simply not reabsorbed in the tubular system—but maintenance of electrical neutrality is a problem with which the body may find it very difficult to cope. In effect, unless the metabolic acid is undissociated at the pH of the urine, an equivalent cation has to be excreted for every anion. This cannot be H^+, because of the limited capacity of the kidneys to excrete an acid urine. The point may be made explicit by reference to sulphate, a normal constituent of urine. Normal urine contains about 50 m Eq/litre of SO_4^{2-}, but one does not suppose that urine is 0.05 N in H_2SO_4. The sulphate ions are balanced by cations, either Na^+ or K^+. This daily occurrence is not a problem, particularly as the intake of both Na^+ and K^+ are usually greater than minimal requirements.

However, in serious metabolic acidosis the provision of cations, whether Na^+, NH_4^+, intercellular K^+, or Ca^{2+} from bone, may become limiting so that renal compensation of acidosis is incomplete. Before outlining this important topic of cations in urine, it is convenient to discuss the capacity of the kidneys to acidify urine.

Excretion of H^+ in Urine

The minimum pH of urine is about 4.5; this is equivalent to 0.03 mEq/litre, a trivial amount. The only way for excretion of H^+ to be increased is by the simultaneous excretion of a Bronsted base—a proton acceptor with a pK of 4.5 or greater. In normal urine the only such base of importance is HPO_4^{2-}. Daily excretion of phosphate is about 50 mEq. The amount of H^+ removed from solution by transfer of phosphate ion (pK 6.8) from an environment of pH 7.4 to one of pH 4.5 is about 80% of the phosphate excreted, *i.e.*, about 40 mEq in a normal person. This is a maximum; if the urine pH is 6 the amount of proton removed is slightly less. All other acid excretion has to be balanced by 'fixed' cations, or by proton uptake by the acid anion itself.

The acids to be found in urine have a wide range of pK's. As already mentioned, H_2SO_4 will always be fully ionized. Lactic acid (pK 3.9) will also be fully ionized, but the second dissociation constant of malic and citric acids (~5) ensures that one —COO^- of each of these acids can act as a proton acceptor in urine. These acids are metabolically unimportant, bit acetoacetic acid (pK 3.6) and 3-hydroxybutyric acid (pK 4.4) can be excreted in considerable amounts in ketosis. Acetoacetic acid will always be fully ionized, but 3-OH butyrate can accept about 0.5 H^+ equivalent per equivalent of acid, at pH 4.5. There is usually more 3-OH butyric than acetoacetic acid in ketotic blood and urine.

There are two Bronsted bases that can appear in urine, which seem at first sight to be suitable acceptors for a significant amount of H^+. These are bicarbonate and ammonia:

(*a*) $H^+ + HCO_3^- \longrightarrow H_2CO_3$ pK 6.1

and base

(*b*) $H^+ + NH_3 \longrightarrow NH_4^+$ pK 9.2

In neither case, however, do these reactions lead to a net loss of H^+ from the kidney. As Fig. 32.9 (*a*) shows, the H_2CO_3 formed in (*a*) returns (as CO_2) to the tubule cells and re-dissociates into H^+ and HCO_3^- there. The bicarbonate is transferred to the blood, but the H^+ remains in the cells.

With (*b*), the proton is retained in the cells before the uncharged ammonia diffuses into the lumen [Fig. 32.9 (*b*)]. Thus for every proton bound in the urine, an equivalent proton is gained by the tubule cells.

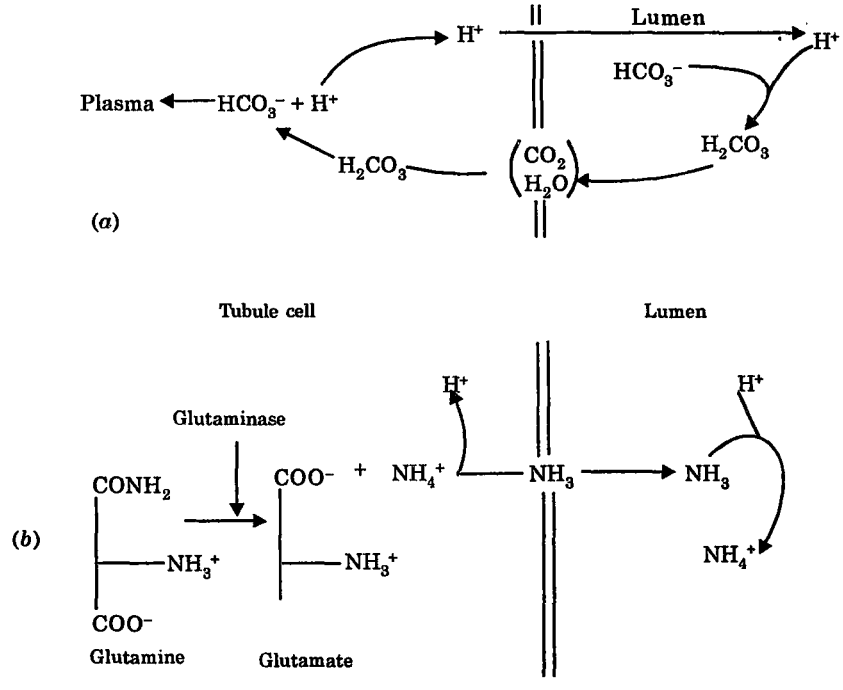


Fig. 32.9. (*a*) Failure of HCO_3^- to act as a proton acceptor in urine. The H_2CO_3 that is formed diffuses back into the tubular cells and re-dissociates, forming the same amount of H^+ there as before. (*b*) Failure of NH_3 to buffer protons in urine. The hydrolysis of glutamine releases NH_4^+ in the tubule cells. For this to pass through the wall of the lumen as NH_3, in must leave a proton behind, which is exactly equivalent to the proton that is taken up in the luminal fluid.

It is nevertheless true that, taking protein catabolism as a whole, the transition from the

It is nevertheless true that, taking protein catabolism as a whole, the transition from the neutral peptide bond —CO—NH— to the neutral molecule urea H_2N—CO—NH_2 involves no net proton changes, while the transition from the peptide bond to the formation (and excretion) of an ammonium ion NH_4^+ results in the formal loss of one H^+ from the system. This approach is unrewarding, however, because it implies that urea and ammonia are alternatives which replace each other according to the acid-base status of the individual. In fact, NH_4^+ formation and excretion is entirely under the control of the kidney, while urea synthesis occurs quite independently in liver, in response to the nitrogen balance of the organism, and can be manipulated independently of the acid-base state. It is more useful to consider NH_4^+ as a slow, but very important, mechanism for conserving cation, confined entirely to the kidneys. This is considered in more detail in the next section.

It must be remembered that we are here considering only the *net* loss to the body of H^+; the total rate of secretion of H^+ into the lumen is very much greater than the net loss.

Alkali Cations in Acidosis

Normal urine contains about 150 mEq/litre Na^+ (and Cl^-) and about 60 mEq/litre K^+. It also contains about 40 mEq/litre NH_4^+, and is slightly acid. The amount of Na^+ and K^+ excreted is very variable, because it depends on the intake (usually in excess of requirements, and on other circumstances, *e.g.*, loss through sweating. With a normal acid-base status, the loss of Na^+ (and Cl^-) can be reduced almost to zero to conserved cation, although there is always a slight loss of K^+ (because there is not plasma-directed K^+ pump).

When the urine becomes more acid than pH 6, the compensatory mechanisms described above come into play. Buffering by phosphate actually conserves Na^+, because of the effective replacement of Na_2HPO_4 (in plasma) by NaH_2PO_4 (in urine), but the excretion of organic anions in metabolic acidosis, or Cl^- in respiratory acidosis necessarily implies the loss of 'fixed' base, which is very largely Na^+. There is only a limited reservoir of Na^+ within the body, and an acute massive acidosis can lead to immediate problems of reduced extracellular fluid volume, increased haematocrit, and so on. In chronic severe acidosis, there is some supplementation by mobilization of Ca^{2+} from bone, K^+ from cells (by replacement with H^+), and even of Mg^{2+}. However, the major relief of Na^+ loss comes from quite a different mechanism.

This involves replacement of Na^+ by NH_4^+ (produced by the hydrolysis of glutamine in the kidney). In general, the mechanism is slow-acting; it takes about 5 days for a significant change to become established, although increased NH_4^+ output is seen after short-term lactic acidosis produced by exercise, or respiratory acidosis induced by re-breathing CO_2. However, the final response, which comes partly from synthesis of new glutaminase protein, can be very large. 500-600 mEq NH_4^+ can be secreted per day, over an indefinite period, and Na^+ excretion can fall well below the normal level. The glutamine required for this mechanism is synthesized in liver, but as mentioned above, the liver does not regulate amino acid group catabolism. A negative N balance is quite common in acidosis.

It should be pointed out that the continued excretion of large quantities of anions, whether neutralized by NH_4^+ or not, does produce a marked diuresis. If the acidosis is a result of uncontrolled diabetes, this is likely to be made worse by glucosuria.

Alkalosis

The body's defences against alkalosis are less effective than against acidosis. This is because the H_2CO_3/HCO_3^- buffer system has less capacity at pH's above 7.4, and also because the urine has very little capacity to buffer OH ions, and indeed no known mechanism for secreting these ions directly. It is fortunate that alkalosis is not so common as acidosis : quite often it is a response to hypokalaemia.

(a) Respiratory response : The respiratory centre is sensitive both to pCO_2 and to pH. A rise in pH will cause under-breathing, which raises the blood pCO_2 so brings the ratio $[HCO_3^-]/[CO_2 + H_2CO_3]$ back to normal. However, under-breathing leads to O_2 deficit and thus cannot be continued indefinitely. The respiratory response to alkalosis is consequently limited.

(b) Renal response : Even if there were to be direct secretion of OH^- into urine (*e.g.*, in exchange for Cl^-), the reaction would ensure that bicarbonate ion would be the apparent product. Thus the response to alkalosis resolves itself into the secretion of an alkaline urine containing HCO_3^-. This has immediate consequences for electrolyte balance, because a cation (usually Na^+) must also be excreted. The excretion of NH_4^+, to conserve Na^+, is not possible because the hydrolysis of glutamine in the kidney is inhibited almost completely by a rise in pH.

Bicarbonate in Urine

The glomerular filtrate contains ca. 25 mM HCO_3^-, but normal urine contains little or no bicarbonate. As the filtrate volume is approximately 200 litres/24 hr, some 5000 mEq of HCO_3^- are removed from urine every day. Most people think that the mechanism is by secretion of H^+, which converts the HCO_3^- and subsequently to CO_2, which diffuses out from the lumen (Fig. 32.9). This would imply that the total excretion of H^+ into the lumen of the tubules would also be of this order of magnitude. The mechanism is for the moment irrelevant; the question is, what controls the appearance of the ion in the final urine ?

Recent research has shown that reabsorption of HCO_3^- is inversely related to the arterial blood volume. If the volume is expanded above normal, reabsorption of HCO_3^- and Na^+ is depressed, HCO_3^- begins to appear in urine, and a threshold maximum concentration of 28 mEq/l in plasma has been observed experimentally.

However, it the blood volume is below normal, no plateau for HCO_3^- reabsorption is seen. The view that there is no absolute plasma maximum value is strengthened by the fact that in respiratory acidosis the plasma $[HCO_3^-]$ may rise to 40 mEq/l, yet no bicarbonate appears in the urine.

If the effects of changes in blood volume are allowed for, it seems that HCO_3^- begins to appear in urine when $[H^+]$ secretion falls off, as a result of rising pH. Although this agrees in a general way with the mechanism depicted in Fig. 32.9 (*a*), the precise relation between pH and H^+ secretion is not known.

Vomiting : Loss of any fluid with a ratio of $[Cl^-]/[HCO_3^-]$ greater than that found in plasma typically gastric juice—will lead to alkalosis. Control by excretion of an alkaline urine may be difficult because the fluid loss will reduce the blood volume (see above).

Hypokalaemia : In part of the renal tubules there is a secretory mechanism in which K^+ and H^+ ions compete with each other. Consequently if the plasma $[K^+]$ is low, more H^+ ions are secreted into the lumen, and the plasma becomes alkaline. It is to be thought that this alkalosis could only be released by administration of K^+, but it has been found that expansion of the extracellular fluid volume with NaCl solution will depress HCO_3^- reabsorption sufficiently to correct the alkalosis.

A frequent cause of potassium alkalosis is increased aldosterone in the blood, which increases the reabsorption of Na^+ and the excretion of K^+.

33 Biochemistry of Muscles

(CONTRACTILE AND STRUCTURAL PROTEINS)

33.1 Introduction

Muscle is the major biochemical transducer (machine) that converts potential (chemical) energy into kinetic (mechanical) energy. Muscle is the largest single tissue in the human body, comprising somewhat less than 25% of body mass at birth, more than 40% of body mass in the young adult, and somewhat less than 30% in the aged adult.

Muscle is only a pulling machine, not a pushing machine. Therefore, a given muscle must be antagonized by another group of muscles or another force such as gravity or elastic recoil.

There are three types of muscle tissues in the body :

1. Striated (Voluntary) or skeletal muscle
2. Non-striated (Involuntary) or smooth muscle
3. Cardiac muscle.

Most skeletal muscle, as the name implies, is attached to bones, and its contraction is responsible for the movements of the skeleton. The contraction of skeletal muscle is controlled by the somatic nervous system and is under voluntary control. The movements produced by skeletal muscle are primarily involved with interactions between the body and the external environment.

Smooth muscle surrounds hollow organs and tubes such as the stomach, intestinal tract, urinary bladder, uterus, blood vessels, and air passages to the lungs. It is also found as single cells distributed throughout organs (*e.g.*, spleen) and in small groups of cells attached to hairs in the skin or the iris of the eye. Contraction of the smooth muscle surrounding hollow organs may propel the luminal contents through the hollow organ (*e.g.*, the intestinal tract), or it may regulate

the flow of the contents through tubes by changing the tube diameter and thus the frictional resistance to flow (*e.g.*, in blood vessels). Smooth-muscle contraction is controlled by the autonomic nervous system, hormones, and factors intrinsic to the muscle itself; it is not usually under direct conscious control.

The third type of muscle, cardiac muscle, is the muscle of the heart, and its contraction propels blood through the circulatory system. Like smooth muscle, it is regulated by the autonomic nervous system, hormones, and factors intrinsic to the muscle itself, it is not usually under direct conscious control.

The third type of muscle, cardiac muscle, is the muscle of the heart and its contraction propels blood through the circulatory system. Like smooth muscle, it is regulated by the autonomic nervous system, hormones, and intrinsic factors in the cardiac muscle.

Although there are significant differences in the structures, mechanical properties, and control mechanisms of the three types of muscle, the force generation mechanism is similar in all of them.

33.2 Structure of Skeletal Muscle Fibres

Skeletal muscle is composed of fibrils surrounded by electrically excitable membrane, the *sarcolemma* (Fig. 33.1) The individual muscle fibre consists of a bundle of many myofibrils arranged in parallel. These are embedded by intramuscular fluid termed the *sarcoplasm*. The fluid contains glycogen, the high energy compounds ATP and phosphocreatine and the enzymes of glycolysis. The functional unit of muscle is *sarcomere*. It exists along the axis of a fibril at distances of 2.5 μm.

Alternating dark and light bands (A bands and I bands) are present in the myofibrils. The central region of the A band (the H zone) is less dense than the rest of the band. The I band is bisected by very dense and narrow Z line.

The cross-section of myofibril under electron microscope shows that myofibrils are constructed of two types of longitudinal filaments. One type confined to the A band (the thick filament) contains mainly the protein *myosin*. These filaments are about 16 nm in diameter. The other filament (the thin filament) lies in the I band but extends also into the A band. The thin filaments are smaller than those of myosin (about 6 nm in diameter).

The thin filaments contain the proteins actin, tropomyosin and troponin. Each thin filament lies between 3 thick filaments.

When muscle contracts, there is no change in the lengths of the thick filaments or of the thin filaments, but the H zone and the I bands shorten. Thus, the arrays of interdigitating filaments must slide past one another during muscle contraction. The cross-bridges generate and sustain the tension. The tension developed during muscle contraction is proportionate to the filament overlap and thereby the number of cross-bridges. Each cross-bridge head is connected to the thick filament via a flexible fibrous segment that can bend outward from the thick filament to accommodate the interfilament spacing.

33.3 Protein Muscles

The muscle fibrils are composed of :

(1) Proteins — 20%.

(2) Water — 75%.

(3) Inorganic materials, certain organic extractives and carbohydrates. (glycogen and its derivatives) –50%.

Muscle proteins are characterized by their elasticity.

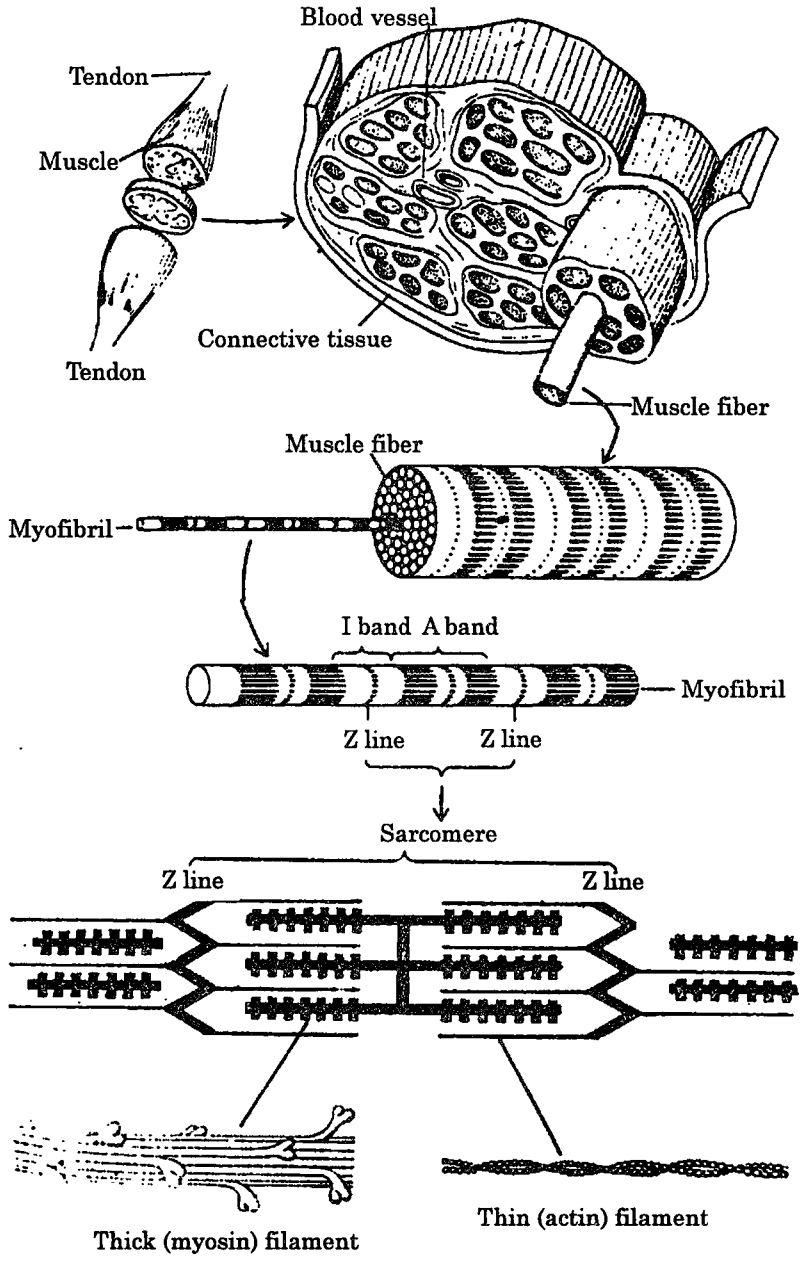


Fig. 33.1. Levels of structural organization in a skeletal muscle.

Myosin

1. Myosin is the richly abundant muscle protein and it is a globulin.
2. It is soluble in dilute salt solutions and insoluble in water.
3. Its molecular weight is 5,00,000 containing 2 major chains and 4 light chains.
4. The enzyme *trypsin* cleaves myosin into two components — *meromyosins* — of unequal size. Therefore, they are termed as *light* and *heavy* meromyosins.

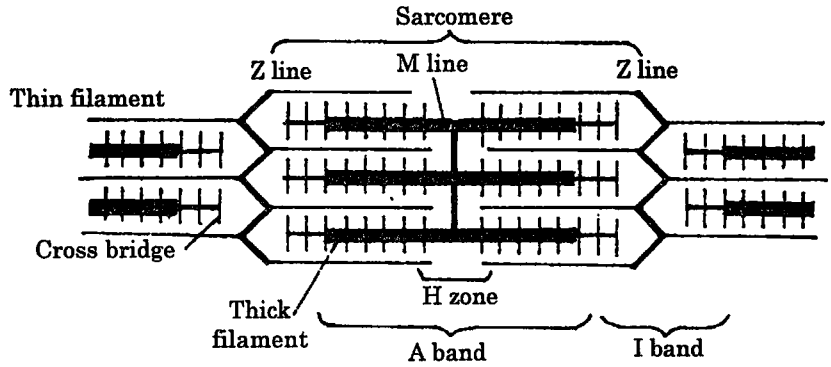


Fig. 33.2. Filament organization of myofibrils. (A) Numerous myofibrils in a single skeletal muscle fiber (arrow indicates mitochondria located between the myo-fibrils). (B) High magnification of a single sarcomere within a single myofibril (arrow indicates end of thick filament). (C) Arrangement of thick and thin filaments that produces the striated banding patterns in myofibrils.

5. It has the adenosine triphosphatase (ATPase) activity.
6. Myosin binds actin forming *actomyosin*. Light meromyosin does not combine with actin. But heavy meromyosin combines with actin.
7. Heavy meromyosin is a rod-shaped protein attached to the two globular components of myosin. The rod portion can be splitt off of the globular region by the action of papain and the resulting portions are termed as HMM (heavy meromyosin) S-2 (the rod) or HMM S-1 (the globular portion). Each HMM S-1 possesses an active site for ATPase activity and a binding site for actin.

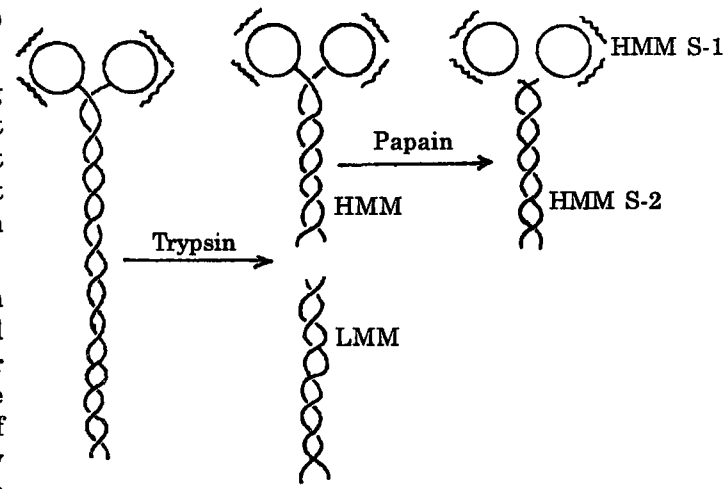


Fig. 33.3. Enzymatic cleavage of myosin.
HMM—Heavy meromyosin.
LMM—Light meromyosin.

8. The 4 light chains of myosin molecule are bound to the HMM S-1 fragments. The light chains function as modulators of ATPase activity.
9. Actomyosin is formed by 3 myosin molecules with 1 actin molecule.

Actin

1. Actin is a globulin of molecular weight 60,000.
2. It is the major constituent of thin filaments in striated muscle.
3. During the preparation of actin by extraction with solution of low ionic strength it is obtained as a molecular weight of 42,000 in a globular configuration called *G-actin*.

4. G actin polymerizes to the fibrous form F-actin in the presence of Mg^{++} and ATP is hydrolysed to ADP and Pi is released. Hence, ATP must be added to set depolymerization of F-actin to G-actin.
5. Actomyosin is formed by actin and myosin. But ATP dissociates to actin and myosin.

$$\text{Actin} + \text{Myosin} \underset{\text{ATP}}{\rightleftharpoons} \text{Actomyosin}$$

Tropomyosin and Troponin

1. Tropomyosin and troponin complex are proteins present in the thin filaments of muscle.
2. Calcium ion has the effect on the interaction of actin and myosin which in turn is mediated by tropomyosin and troponin.
3. Tropomyosin is a double-stranded α-helical rod of molecular weight 70,000 and present between the 2 strands of F actin.
4. Troponin is a complex of 3 polypeptide chains—TPC, TPI, TPT.
5. The troponin complex is present in the thin actin filaments at intervals of 38.5 nm.
6. A troponin complex with tropomyosin molecule regulates the activity of 7 actin monomers.

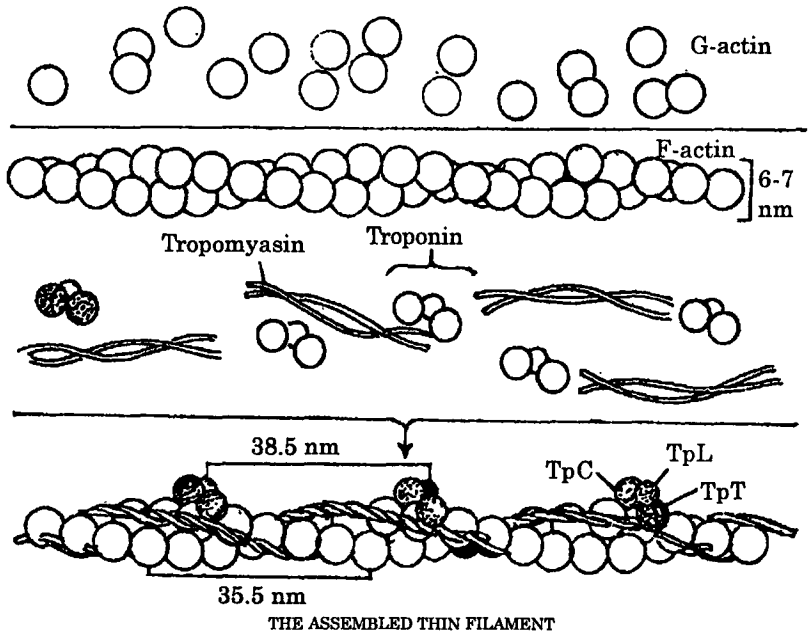


Fig. 33.4. Schematic representation of the thin filament, showing the spatial configuration of the 3 major protein components—actin, tropomyosin, and troponin.

33.4 Molecular Mechanisms of Contraction

Sliding-Filament Mechanism

The lengths of neither the thick filaments nor the thin filaments change during shortening; rather, the two sets of filaments slide past each other. The following observations led to the sliding-filament mechanism of muscle contraction. When a skeletal muscle fiber shortens, the width of the A band in a sarcomere remains constant (Fig. 33.5). Since this width corresponds to the length of the thick filaments, these filaments do not change length during muscle shortening. In contrast, the widths of both the I band and the H zone decrease during shortening as the thin filaments

move past the thick filaments. The H zone decreases as the ends of the thin filaments from opposing ends of a sarcomere approach each other, and it disappears when these thin filaments meet in the center of the A band. The I band also decreases in width for the same reason, *i.e.*, as more and more of the thin filament length overlaps the thick filaments. During shortening the length from the edge of the H zone to the nearest Z line (the length of a thin filament) remains constant. Thus, the lengths of the thin filaments also remain constant during shortening.

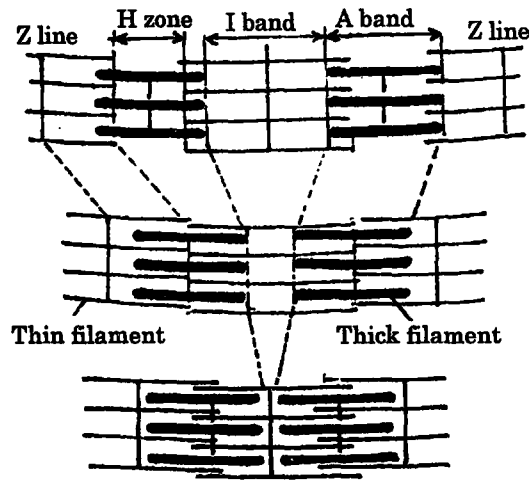


Fig. 33.5. Changes in filament alignment and banding pattern in myofibril during shortening.

What produces the movement of these filaments ? The answer is *the cross-bridges.* When a cross-bridge is activated, it moves in an arc parallel to the long axis of the thick filament, much like an oar on a boat. If, at this time, the cross-bridge is attached to a thin filament, this swivelling motion slides the thin filament toward the centre of the A band, thereby producing shortening of the sarcomere. One stroke of a cross-bridge produces only a small displacement of a thin filament relative to a thick filament, but the cross-bridges undergo many cycles of movement during a single contraction each cycle requiring attachment of the bridge to the thin filament, angular movement of the bridge while attached, detachment from the thin filament, reattachment at a new location, and repetition of the cycle. Each cross-bridge undergoes its own independent cycle of movement, so that at any one instant during contraction only about 50 per cent of the bridges are attached to the thin filaments, while the others are at intermediate stages of a cycle.

Let us look more closely at the filaments and their cross-bridges. Molecules of actin are arranged in two chains, helically intertwined to form the thin filaments (Fig. 33.6). Myosin is a much larger molecule with a globular end attached to a long tail. Approximately 200 myosin molecules comprise a single thick filament; the tails of the molecules lie along the axis of the filament and the globular heads extend out to the sides, forming the cross-bridges. Each globular head contains a binding site able to bind to a complementary site on an actin molecule. The myosin molecules in the two halves of each thick filament are oriented in opposite directions, such that all their tail ends are directed towards the centre of the fiber (Fig. 33.7). Because of this arrangement, the power strokes of the cross bridges in the two ends of each thick filament are directed toward the centre, thereby moving the thin filaments at both ends of the sarcomere toward the centre of the sarcomere during shortening.

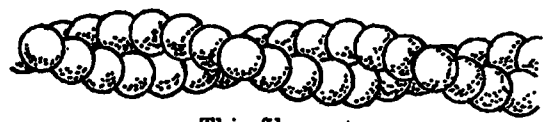


Fig. 33.6. Two helical chains of globular actin molecules form the primary structure of the thin filaments.

In addition to the binding site for actin, the globular end of myosin contains a separate enzymatic site that catalyzes the breakdown of ATP (adenosine triphosphate) to ADP (adenosine diphosphate) and inorganic phosphate, releasing the chemical energy stored in ATP. The splitting

of ATP occurs on the myosin molecule before it attaches to actin, but the ADP and inorganic phosphate generated remain bound to myosin. The chemical energy released at the time of ATP splitting is transferred to myosin (M), producing an energized form of myosin (M*).

$$M + ATP \xrightarrow[\text{ATP breakdown}]{} M^*.ADP.P_i$$

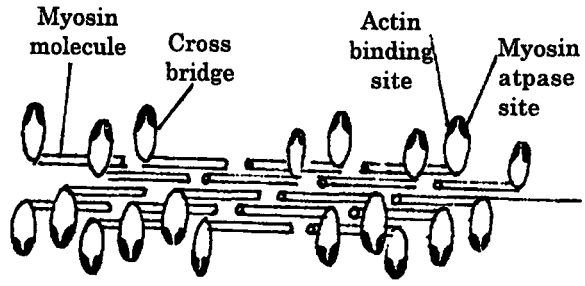


Fig. 33.7. Orientation of myosin molecules in thick filaments, with the globular heads of the myosin molecules forming the cross-bridges.

The subsequent binding of this energized myosin to actin (A) via a cross-bridge triggers the discharge of the energy stored in myosin. With the resulting production of the force that causes movement of the cross bridge (the ADP and P_i are released from myosin at this time) :

$$A + M^*.ADP.P_i \xrightarrow[\text{Action binding}]{} A.M^*.ADP.P_i \xrightarrow[\text{Energy release and bridge movement}]{} A.M + ADP.P_i$$

This sequence of energy storage and release by myosin is analogous to the operation of a mousetrap. Energy is stored in the trap by cocking the spring (by ATP splitting) and released by springing the trap (by binding to actin). This is, of course, only an analogy; the actual structural changes in myosin which accompany energy storage and release are unknown.

During contraction, the myosin cross-bridge binds very firmly to actin, and this linkage must be broken at the end of each bridge cycle; the binding of a new molecule of ATP to myosin is responsible for breaking this link :

$$A.M. + ATP \xrightarrow[\text{A.M. dissociation}]{} A + M.ATP$$

Thus, upon binding (but not splitting) a molecule of ATP, myosin dissociates from actin. The free myosin bridge then splits its bound ATP, thereby re-forming the energized state of myosin, which can now reattach to a new site on the actin filament and so on. Thus, ATP performs two distinct roles in the cross-bridge cycle : (1) The energy released from the splitting of ATP provides the energy for cross-bridge movement; and (2) the binding (not splitting) of ATP to myosin breaks the link between actin and myosin at the end of a cross-bridge cycle allowing the cycle to be repeated. Figure 33.8 provides a summary of these chemical and mechanical changes that occur during one cross-bridge cycle. Many cross-bridge cycles occur during even the briefest contraction of a muscle fiber.

The importance of ATP in dissociating actin and myosin at the end of a bridge cycle is illustrated by the phenomenon of rigor mortis (death rigor) the stiffening of skeletal muscles after death. Rigor mortis begins 3 to 4 *h* after death and is complete after about 12 *h*, but then slowly disappears over the next 48 to 60 *h*. Rigor mortis occurs because of the lack of ATP. In the absence of ATP the myosin cross-bridges are still able to bind to actin, but the subsequent breakage of the link between them and the energizing of myosin requires ATP, which is not produced in a dead muscle cell. The thick and thin filaments become linked to each other by the immobilized cross-bridges, producing the rigid connection of a dead muscle. In contrast, in a living muscle at rest, the myosin bridges are not bound to actin, and the filaments readily slide past each other when the muscle is passively stretched.

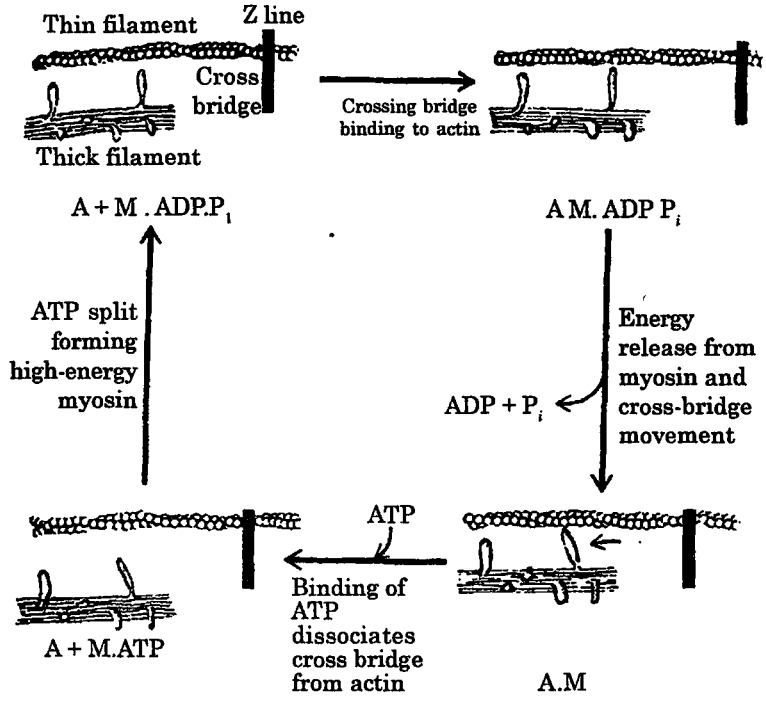


Fig. 33.8. Chemical and mechanical changes during the four stages of single cross-bridge cycle. Start reading the figure at the lower left.

33.5 Muscle Energy Metabolism

The ATP required as the constant energy source for the contraction-relaxation cycle of muscle can be generated by glycolysis, oxidative phosphorylation, creating phosphate, or two ADP molecules. The ATP stores in skeletal muscle are short-lived during contraction, providing energy probably for less than 1 second of contraction.

Phosphagens such as creatine phosphate prevent the rapid depletion of ATP by providing a readily available high-energy phosphate, which is all that is necessary to reform ATP from ADP. Creatine phosphate is formed from ATP and creatine at times when the muscle is relaxed and ATP demands are not so great. The enzyme catalysing the phosphorylation of creatine is creatine phosphokinase (CPK), a muscle-specific enzyme with clinical utility in the detection of acute or chronic disorders of muscle.

Skeletal muscle sarcoplasm contains significant glycogen stores. The release of glucose from glycogen is dependent upon a specific muscle phosphorylase enzyme. This enzyme is missing in a specific disorder of muscle (McArdle's disease), a form of glycogen storage disease.

ATP is also available from oxidative phosphorylation in muscle tissue, a process dependent upon a constant oxygen supply. Muscles that have high oxygen demands as a result of sustained contraction (such as to maintain posture) have the ability to store oxygen in *myoglobin*. Because of the heme moiety to which oxygen is bound in myoglobin, muscles containing myoglobin are red, as compared to white skeletal muscle. Table 33.1 compares some of the properties of fast or white skeletal muscle with slow or red skeletal muscle.

Table 33.1

CHARACTERISTICS OF FAST AND SLOW SKELETAL MUSCLES

	Fast Skeletal Muscle	*Slow Skeletal Muscle*
Myosin ATPase	High	Low
Energy utilization	High	Low
Colour	White	Red
Myoglobin contraction	No	Yes
Rate	Fast	Slow
Duration	Short	Prolonged

Myoadenylate kinase, an enzyme present in muscle, catalyzes the formation of one ATP molecule and one AMP from two ADP molecules. This reaction is shown in Fig. 33.9 coupled with hydrolysis of ATP by myosin ATPase during muscle contraction. The relationships between these various sources of ATP and its consumption during muscle contraction are also depicted.

In humans, skeletal muscle protein is the major nonfat source of stored energy. This explains the very large losses of muscle mass, particularly in adults, resulting from prolonged caloric undernutrition.

The study of tissue protein breakdown *in vivo* is difficult because amino acids released during intracellular breakdown of proteins can be extensively reutilized for protein synthesis within the cells, or the amino acids may be transported to other organs where they enter anabolic pathways. However, actin and myosin are methylated following synthesis of their peptide bonds, producing *3-methylhistidine* (3-MeHis). During intracellular breakdown of actin and myosin, 3-MeHis is released and excreted into the urine. When labelled material was administered to rats and humans, it was found that the urinary output of the methylated amino acid provides a reliable index of the rate of myofibrillar protein breakdown in the musculature of rats of human subjects. The fractional rate of muscle protein breakdown is not significantly different in the elderly as compared with young adults, but since muscle mass is less in the elderly, this tissue contributes less to the whole body protein breakdown that occurs with aging in humans.

As noted above, skeletal muscle is the major reserve of protein the body. In addition, this tissue is highly active in the degradation of certain amino acids as well as in the synthesis of others. In mammals, muscle appears to be the primary site of catabolism of the branched chain amino acids. It oxidizes leucine to CO_2 and converts the carbon skeletons of aspartate, asparagine, glutamate, isoleucine, and valine into intermediates of the tricarboxylic acid cycle. The capacity of muscles to degrade branched chain amino acids increases 3-to 5-fold during fasting and in diabetes.

Muscle also synthesizes and releases large amounts of alanine and glutamine. These compounds are synthesized utilizing amino groups that are generated in the breakdown of branched chain amino acids, and nitrogen is then transferred to α-ketoglutarate and to pyruvate by transamination. Glycolysis from exogenous glucose is the source of almost all of the pyruvate for synthesis of alanine. These reactions constitute the so-called "glucose-alanine cycle", wherein alanine from muscle is utilized in hepatic gluconeogenesis while at the same time bringing amino groups to the liver for removal as urea.

The carbon skeletons of the amino acids that are degraded in muscle and enter the tricarboxylic acid cycle in muscle are converted mostly to glutamine and to pyruvate, which itself is further oxidized or converted to lactate. It thus appears that in fasting or the postabsorptive

state, muscle releases most amino acids coming from net protein breakdown except for isoleucine, valine, glutamate, aspartate, and asparagine, which are used to contribute to the formation of glutamine, which itself is released for use by other tissues.

For many years, it has been observed that working muscle releases ammonia. It is now known that the immediate source of ammonia in skeletal muscle is AMP, which is deaminated to IMP, catalyzed by adenylate deaminase. IMP may be converted back to AMP by reactions utilizing aspartate and catalyzed by adenylosuccinate synthetase and adenylosuccinase.

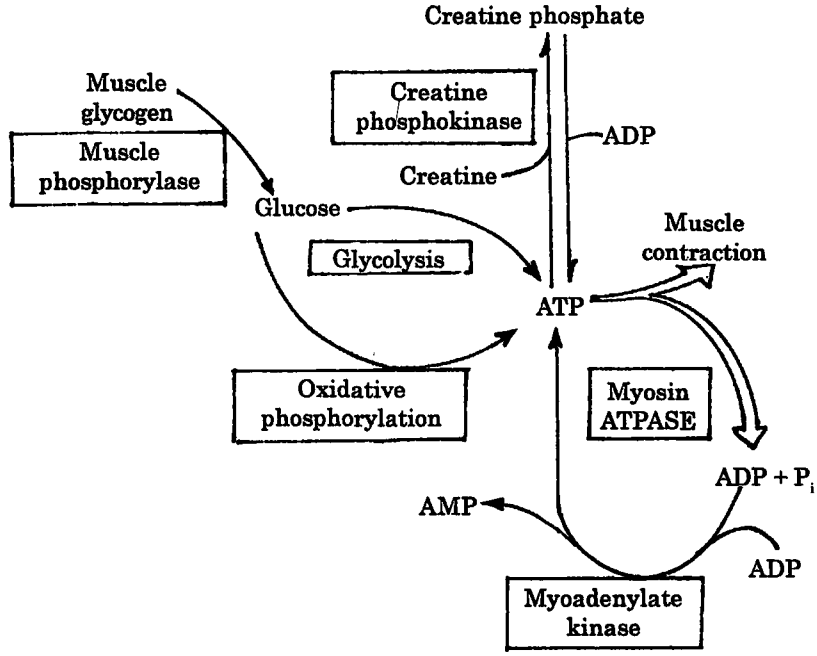


Fig. 33.9. The multiple sources of ATP in muscle.

33.6 Muscle Fatigue

When a skeletal muscle fiber is continuously stimulated at a frequency that produces a maximal tetanic contraction, the tension developed by the fiber eventually declines. This failure of a muscle fiber to maintain tension as a result of previous contractile activity is known as *muscle fatigue*. Figure 33.9 illustrates several characteristics of the fatigue process. Fatigue is not limited to tetanic contractions but can occur with twitches if they are repeated frequently enough over an extended period of time. On the other hand, low-frequency twitches can be repeated indefinitely in certain types of skeletal muscles with no signs of fatigue. Thus, the onset of fatigue and its rate of development depend on the type of skeletal muscle and the intensity of contractile activity.

If, after the onset of fatigue, a muscle is allowed to rest for a period of time, it can recover its ability to contract upon restimulation. The extent of recovery after a period of rest varies depending upon the type of previous activity. A muscle will fatigue fairly rapidly if stimulated at a high frequency, but it will also recover rapidly from this fatigue. This is type of fatigue that accompanies short-duration, high-intensity types of exercise, such as weight lifting. On the other hand, fatigue that develops more slowly with long-duration, low intensity endurance exercise, such as long distance running, requires much longer periods of rest, often upto 24 *h* before complete recovery is achieved.

It might seem logical that depletion of available ATP would account for fatigue, but it does not. The ATP concentration in fatigued muscle is only slightly lower than in a resting muscle. Indeed it is neither the supply of energy not the ability of the contractile proteins to generate tension that is responsible for fatigue. Rather, a failure of some step in excitation-contraction coupling appears to be the most likely cause of fatigue, but which step and what brings about its failure is currently unknown. Failure of the appropriate levels of the cerebral cortex to send excitatory signals to the motor neurons of a particular muscle can lead to its failure to contract. This type of *psychological fatigue* causes an individual to stop exercising even though the muscles themselves are not fatigued. An athlete's performance depends not only on the physical state of the appropriate muscles but also upon the "will to win", the ability to overcome psychological fatigue.

33.7 Contraction of Whole Muscles

The human body contains over 600 different skeletal muscles, which taken all together make up the largest tissue in the body, accounting for 40 to 45 per cent of the body weight. Some muscles are very small consisting of only a few hundred fibres; larger muscles may contain several hundred thousand fibers.

Surrounding the individual muscle fibres in a muscle is a network of collagen fibres and connective tissue through which pass blood vessels and nerves. Collagen is a fibrous protein which has great strength but no active contractile properties. Each end of a muscle is usually attached to bone by bundles of collagen fibers known as tendons. The collagen fibres surrounding the fibres and in the tendons act as a structural framework which transmits the muscle tension to the bones.

In some muscles, the individual fibres extend the entire length of the muscle, but in most, the fibres are shorter. When fibres are shorter, their ends are anchored to the connective tissue network within the muscle. The transmission of force from muscle to bone is like a number of people pulling on a rope, each person corresponding to a single muscle fibre and the rope corresponding to the connective tissue and tendons.

Some tendons are very long, and the site of attachment of the tendon to bone is far removed from the muscle. For example, some of the muscles which move the fingers are in the forearm, as one can observe by wiggling one's fingers and feeling the movement of muscles in the lower arm. These muscles are connected to the fingers by long tendons. If the muscles which move the fingers were located in the fingers themselves, we would have very fat fingers.

33.8 Collagen

Collagen, the major macromolecule of connective tissues, is the most common protein in the animal world. It provides an extracellular framework for all metazoan animals and exists in virtually every animal tissues. There are at least 5 distinct types of collagen in mammalian tissues; thus, they exist as a family of molecules sharing many properties.

The most definitive property of collagen molecules is their triple helix, a coiled coil of 3 polypeptide subunits. Each polypeptide subunit or alpha chain is twisted into a left handed helix of 3 residues per turn. Three of these left-handed helices are then wound to a right handed superhelix to form a stiff rodlike molecule 1.4 nm in a diameter and about 300 nm long. These triple helical molecules—unique to collagen—are then associated bilaterally and longitudinally into fibrils (Fig. 33.10). The arrangement of collagen fibrils involves longitudinal staggering slightly less than one-quarter the length of the triple helix. Between the end of one triple helix and the beginning of the next is a gap that may provide a site for deposition of hydroxyapatite crystals in bone formation. Collagen fibrils range from 10 to 100 nm in diameter and are visible by microscope as banded structures in the extracellular matrix of connective tissues.

The other striking characteristic of the collagen molecule is that glycine constitutes every third residue in the triple helical portion of each alpha chain. Glycine is the only amino acid small enough to exist in the limited space available down the central core of the triple helical molecule; thus, the central core of the triple helical molecule consists of glycine residues provided by each of the 3 alpha subunits. This repeating structure can be represented by $(Gly\text{-}X\text{-}Y)_n$, where X and Y are amino acids other than glycine.

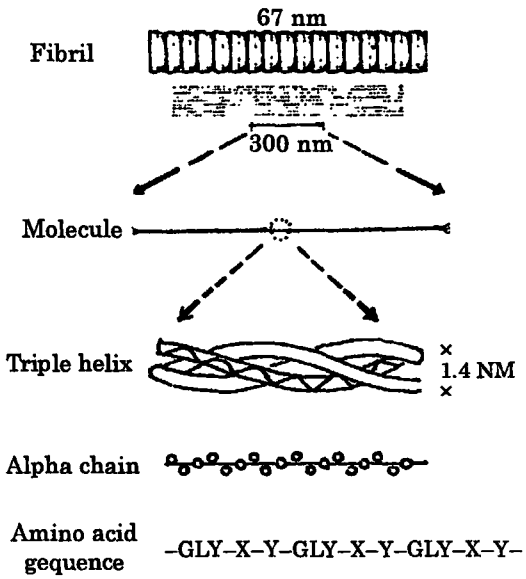


Fig. 33.10. Molecular features of collagen structure from primary sequence up to the fibril.

In mammalian collagen, about 100 of the X positions are *proline* and 100 of the Y positions are *4-hydroxyproline*. These rigid imino acids limit rotation of the polypeptide backbone and thus increase the stability of the triple helix. The hydroxyproline residues contribute additional stability to the collagen triple helix by forming more intramolecular hydrogen bonds mediated through extra water molecules. Collagen also contains *3-hydroxyprolone in some X positions* and *5 hydroxylysine in Y positions. The collagen triple helix is stabilized by multiple interchain cross-links* between lysyl and hydroxylysyl residues. The chemical nature of these cross-links is described below. Mature collagen is a glycoprotein containing saccharides attached in O-glycosidic linkage to the hydroxylysine residues.

A summary of the vertebrate collagens, their tissue distributions, and distinctive features is presented in Table 32.2.

Synthesis of Collagen

Collagen is an extracellular protein but is synthesized as an intracellular precursor molecule that undergoes post-translational modification before becoming a mature collagen fibril. Like all secreted proteins, the precursor of collagen is processed as it passes through the endoplasmic reticulum and Golgi complex prior to appearing extracellularly. The earliest collagen precursor is a *preprocollagen* that contains a leader or signal sequence of approximately 100 amino acids at its amino terminus. Preprocollagen is generated by ribosomes attached to the endoplasmic reticulum. As the signal sequence penetrates into the vesicular space of the endoplasmic reticulum, the leader sequence is cleaved off and the amino-terminal end of *procollagen* continues to protrude into the endoplasmic reticulum space. At this site *prolyl 4-hydroxylase* and *lysyl hydroxylase* act on proline or lysine residues, respectively, in the Y position of the $(Gly\text{-}X\text{-}Y)_n$ peptide. A prolyl 3-hydroxylase acts on prolyl residues in the X position immediately preceding 4-hydroxyproline in the Y position.

The procollagen molecule contains at its amino terminus a 20,000-MW peptide and at its carboxy terminus a 30 to 35 thousand-MW peptide, neither of which is present in mature collagen. Both of these propetides contain cysteine residues. While the amino-terminal propeptide collagen forms only intrachain disulfide bonds, the carboxy terminal peptides form both intrachain and interchain disulfide bonds. Following the formation of these disulfide bonds, the procollagen molecules assemble as the triple helix.

Table 33.2

GENETICALLY DISTINCT VERTEBRATE COLLAGENS. AT LEAST 5 DIFFERENT MOLECULES CONTAINING 7 GENETICALLY DISTINCT a CHAINS ARE PRESENT IN HIGHER ANIMALS

Type	*Molecular Formula*	*Native Polymer*	*Tissue Distribution*	*Distinctive Features*
I	$[\alpha 1(I)]_2\alpha^2$	Fibril	Skin, tendon, bone, dentin, fascia; widespread.	Low content of hydroxylysine; few sites of hydroxylysine glycosylation; broad fibrils.
II	$[\alpha 1(II)]_3$	Fibril	Cartilage, nucleus pulposus, notochord, viterous body.	High content to hydroxylysine; heavily glycosylated; usually thinner fibrils than type 1.
III	$[\alpha 1(III)]_3$	Fibril	Skin, uterus, blood vessels; "reticulin" fibers generally.	High content of hydroxyproline; low content of hydroxylysine; few sites of hydroxylysine glycosylation; interchain disulfides between cysteines at the carboxyl end of the helix; long carboxyl telopeptide.
IV	$[\alpha 1\ (IV)]_3$ (tentative, under dispute)	Basement lamina	Kidney glomeruli, lens capsule; Descemet's membrane; basement laminae of all epithelial and endothelial cells ?	Very high content of hydroxylysine; almost fully glycosylated; relatively rich in 3 hydorxyproline; low alanine content; retains procollagen extension pieces.
V	$\alpha(A(\alpha B)_2$ or $(\alpha A)_3$and $(\alpha B)_3$	Unknown	Widespread in small amounts; basement lamina of smooth and striated muscle cells ? exoskeleton of fibroblasts and other mesenchymal cells ?	High content of hydroxylysine; heavily glycosylated; low alanine content; fails to form native fibrils *in vitro*.

After formation of triple helix, further hydroxylation of prolyl and lysyl residues *cannot* occur. The glycosyltransferase activities that attach glucose or galactose to hydroxylysine residues also require that the procollagen alpha chains be non-helical.

Following this intracellular processing, the glycosylated procollagen molecule reaches the outside of the cell by way of the Golgi complex. Extracellular procollagen aminoprotease and procollagen carboxy protease remove the amino-terminal and carboxy-terminal propeptides, respectively. The newly formed collagen molecules have approximately 1000 amino acids and spontaneously assemble into collagen fibrils that are indistinguishable from the mature fibrils found in tissues.

These fibrils, however, do not have the tensile strength of mature collagen fibrils until they are *crosslinked by a series of covalent bonds.* The extracellular copper-containing enzyme lysyl oxidase oxidatively deaminates the ε-amino groups of certain lysyl and hydroxylysyl residues of collagen, yielding reactive aldehydes. The aldehydes can form Schiff bases with amino groups of other lysines or hydroxylysines or even glycosylated hydroxylysines. These Schiff bases are chemically rearranged and provide stable covalent cross-links such as new peptide bonds or secondary amine bridges. The aldehyde component derived from a hydroxylysine forms a more stable cross-link than does the aldehyde derived from a lysyl residue. Aldol bridges also provide intramolecular cross-links.

The intracellular and extracellular processing of the collagen precursor molecule is summarized in Table 33.3.

Table 33.3

ORDER AND LOCATION OF PROCESSING THE COLLAGEN PRECURSOR (CONTAINING REPEATING STRUCTURE [GLY-X-Y]$_n$)

Intracellular (endoplasmic reticulum)
(1) Cleavage of signal peptide.
(2) 4-Hydroxylation of Y-prolyl residues.
(3) 3-Hydroxylation of X-prolyl where, Y = 4-hydroxyprolyl residue.
(4) 5-Hydroxylation of Y-lysyl residues.
(5) Glycosylation of hydroxylysyl residues.
(6) Formation of intrachain and interchain S-S bonds.
Extracellular
(1) Cleavage of NH_2-terminal propetide (MW 20,000).
(2) Cleavage of COOH-terminal propeptide (MW 30-35,000).
(3) Formation of immature collagen fibrils.
(4) Oxidation of lysyl, hydroxylysyl, glycosylated hydroxylysyl residue to aldehydes.
(5) Cross-linking of chains and helical molecules of fibrils via Schiff bases and aldol condensations.

33.9 Recording of Isotonic and Isomeric Contractions

Figure 33.11 illustrates the general method of recording isotonic and isometric contractions. During an isotonic contraction the distance the muscle shortens, as a function of time, is recorded. The distance moved can be directly measured by a pen that is attached to a muscle (or to muscle lever) and leaves a trace on a moving strip of paper as the muscle contracts and relaxes. During an isometric contraction, the force (tension) generated by the muscle as a function of time is measured. To measure an isometric contraction the muscle is attached at one end to a rigid support

and at the other to a force transducer, which controls the movement of a recording pen in proportion to the force exerted. The terminology and methods of recording contractions described above apply to both single fibres and whole muscles.

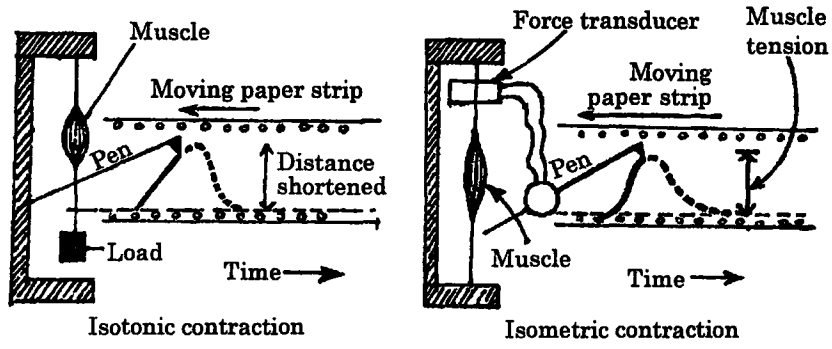


Fig. 33.11.

Twitch Contractions

The mechanical response of a muscle fibre to a single action potential is known as a twitch. Figure 33.12 shows the main features of an isometric and an isotonic *twitch*. In an isometric twitch there is an interval of a few milliseconds, the *latent period,* before the tension begins to increase. It is during this latent period that the processes associated with excitation-contraction coupling are occurring. The time interval from the beginning of tension development to the peak tension is the *contraction time.* Not all skeletal muscle fibres have the same contraction times. Some fast fibres have contraction times as short as 10 ms, whereas slower fibres may take 100 ms or longer. The time from peak tension until the tension has decreased to zero is known as the *relaxation time.*

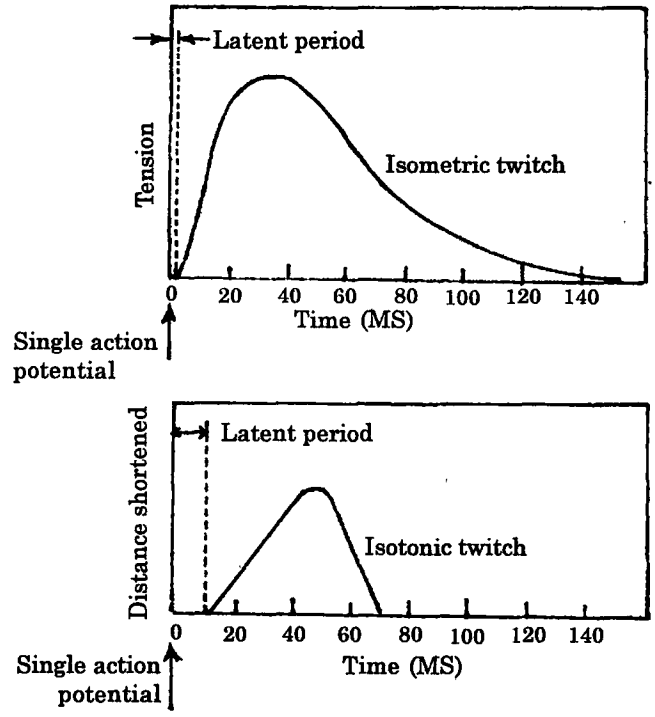


Fig. 33.12. Isometric and isotonic skeletal muscle-fibre twitches following a single action potential.

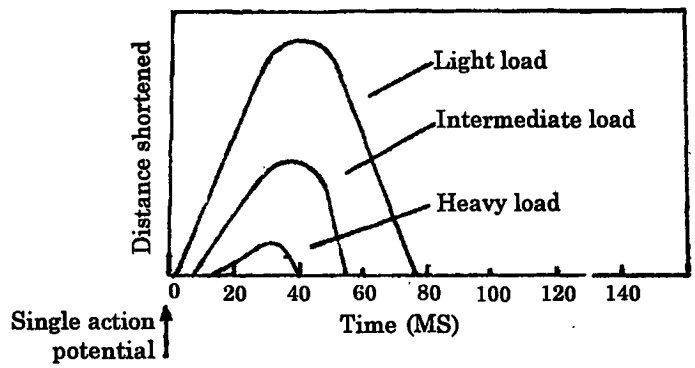


Fig. 33.13. Changes in the isotonic shortening of a muscle fibre with different loads during a twitch.

Comparing an isometric twitch with an isotonic twitch in the same muscle fibre, one can see from Fig. 33.12 that the latent period is longer, whereas the duration is shorter, in an isotonic twitch depend upon the magnitude of the load being lifted (Fig. 33.13). At heavier loads the latent period is longer but the velocity of shortening, the duration of the twitch, and the distance shortened all decrease. During the latent period of an isotonic contraction, the cross-bridges begin to develop force, but actual shortening does not begin until the muscle tension slightly exceeds the load; therefore, the heavier the load, the longer the latent period. If the load on a fibre is increased, eventually a load will be reached that the muscle is unable to lift, the velocity of shortening will be zero, and the contraction becomes isometric.